Characterization of altered cytokine production by memory CD4 T cells in NZBxW murine model of SLE

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Zusammenfassung

Der systemische Lupus erythematodes (SLE) ist eine Autoimmunerkrankung, bei der eine Vielzahl an Organen betroffen sein kann. Hierbei spielen T-Zellen mit gestörter Zytokinproduktion, insbesondere von IL-2 und IFN-γ, eine besondere Rolle. Mit Fortschreiten der Krankheit sinkt die Anzahl an IL-2-Produzenten und gleichzeitig steigt die Anzahl an IFN-γ-Produzenten. Während die Rolle von IFN-γ in SLE bisher kontrovers diskutiert wird, wirkt sich die verringerte Produktion von IL-2 beispielsweise negativ auf regulatorische T-Zellen aus, was zur Pathogenese der Krankheit beiträgt.

In dieser Arbeit erfolgte eine Charakterisierung der Zytokinproduzierenden $CD4^+$ Gedächtnis-T-Zellen in erkrankten NZBxW Mäusen, einem Modell für SLE. Anhand der Produktion von IL-2 und/oder IFN-γ wurde dabei in DN (IFN-γ^-IL-2^-doppelt negative), IL-2 SP (IFN-γ^-IL-2^+ einzelpositive), DP (IFN-γ^+IL-2^+ doppelt positive) und IFN-γ SP (IFN-γ^+IL-2^-einzelpositive) Zellen unterschieden. Ein mehrstufiges Verfahren der Zellsortierung ermöglichte die Isolierung der vier Zellpopulationen. Genexpressionsanalysen legten offen, dass die während der Krankheit vermehrt vorkommende Population der IFN-γ SP Zellen im Vergleich zu DP Zellen deutliche Unterschiede in ihrem Genexpressionsmuster aufweist. IFN-γ SP Zellen exprimieren u.a. verstärkt Chemokinrezeptoren, co-stimulatorische und co-inhibitorische Moleküle, sowie Apoptose-Marker und zeigen eine verminderte Produktion von Effektorzytokinen. Weiterführende funktionelle Analysen untermauerten die Expressionsdaten und zeigten eine verminderte Proliferationsfähigkeit und verstärkte Apoptose der IFN-γ SP Zellen. Die Daten zeigen, dass der Phänotyp der IFN-γ SP Zellen in erkrankten NZBxW Lupus-Mäusen gestört ist, wodurch die IFN-γ SP Zellen zur Erkrankung beitragen könnten.

Summary

Systemic lupus erythematosus (SLE) is an autoimmune disease, which can affect almost every organ system of the body. Thereby altered cytokine production by T cells plays an important role in the pathogenesis of the disease. With disease progression, production of IL-2 decreases and production of IFN-γ increases. It has been shown that IL-2 deficiency affects T_{REG} homeostasis in SLE and thus contributes to its pathogenesis. The role of IFN-γ in SLE is, however, controversial.

In this work, a comprehensive characterization of four subpopulations of memory CD4 T cells of diseased NZBxW lupus-prone mice was performed. These cell subsets are DN (IFN-γ^{-}IL-2^{-} double negative), IL-2 SP (IFN-γ^{-}IL-2^{+} single positive), DP (IFN-γ^{+}IL-2^{+} double positive) and IFN-γ SP (IFN-γ^{+}IL-2^{-}single positive) cells. A multi-step cell sorting procedure was used to isolate these cell subsets. The data showed that IFN-γ SP cells were characterized by a different gene expression profile than DP cells. In detail, IFN-γ SP cells revealed an enhanced expression of chemokine receptors, co-stimulatory and co-inhibitory molecules as well as apoptosis markers and decreased production of effector cytokines. In addition, functional analyses showed that IFN-γ SP cells were tended to increased apoptosis and decreased proliferation. These data show an altered phenotype of IFN-γ SP cells of diseased NZBxW lupus-prone mice, which might be important for the disease pathogenesis at least in this animal model of SLE.

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Abbreviations

A	Adenine
ANA	Anti-nuclear autoantibody
AP-1	Activator protein 1
APC	Antigen presenting cell
APC	Allophycocyanin
ASCT	Autologous stem cell transplantation
B220	B cell isoform of 220 kDa
BAFF	B cell activating factor
BALB/c	Albino laboratory-bred strain of the house mouse
BCL	B cell leukemia/lymphoma
BCR	B cell receptoe
BLyS	B lymphocyte stimulator
BP	Biological process
BSA	Bovine serum albumin
C	Cytosine
CCL	Chemokine (C-C Motif) ligand
CCR	Chemokine (C-C Motif) receptor
CCRL	Chemokine (C-C Motif) receptor-like
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein *N*-succinimidyl ester
CIA	Collagen induced arthritis
CSF	Colony stimulating factor
CSV	Comma-separated values
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CXCR	Chemokine (C-X-C Motif) receptor
DAPL-1	Death-associated protein-like1
DAVID	Database for annotation, visualization and integrated discovery
DN	IFN-γ^{-}IL-2^{-} double negative cells
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DP	IFN-γ^{+}IL-2^{+} double positive cells
dsDNA	Double stranded DNA
EAE	Experimental autoimmune encephalomyelitis
F1	Filial 1
FACS	Fluorescent-activated cells sorting
Fc-γR	Fragment crystallizable gamma receptor
FITC	Fluoresceinisothiocyanat
FOXP3	Forkhead box P3
G	Guanine
GATA-3	GATA Binding Protein 3
GO	Gene ontology
GZMB	Granzyme B
ICOS	Inducible T cell co-stimulator
IFN	Interferon
IFNGR	Interferon gamma receptor
IFN-γ SP	IFN-γ^{+}IL-2^{-} single positive cells
Ig	Immunoglobulin
IL	Interleukin
IL12RB2	Interleukin 12 receptor beta 2
IL18R1	Interleukin 18 receptor 1
IL-2 SP	IFN-γ^{-}IL-2^{+} single positive cells
IL4RA	Interleukin 4 receptor alpha
IRF	Interferon regulatory factor
LAG-3	Lymphocyte-activation gene 3
LN	Lupus nephritis
LOG2	binary logarithm
mAb	Monoclonal antibody
MAC-1	Macrophage-1 antigen
MACS	Magnetic-activated cells sorting
MAPK	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor of κB
NK	Natural killer cell
NKT	Natural killer T cell
NZBxW	New Zeeland Black and White
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCC	Pearson Correlation Coefficient
PCoA	Principal Coordinates Analysis
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
PI	Propidium iodide
PKC	Protein kinase C
PM	Perfect match
PMA	Phorbol 12-myristate 13-acetate
qRT-PCR	Quantitative real-time PCR
RBC	Red blood cell
RIN	RNA integrity numbers
RMA	Multichip averaging
RNA	Ribonucleic acid
RNase	Ribonuclease
RORγT	Retinoic acid receptor-related orphan receptor-γt
RPMI	Roswell Park Memorial Institute
RPS18	Ribosomal protein S18
RT	Room temperature
SATB-1	Special AT-rich sequence-binding protein 1
SLE	Systemic lupus erythematosus
SMAD2	Sma and Mad related proteins
STAT	Signal transducers and activators of transcription
T	Thymidine
T-bet	T-box 21
T_C	Cytotoxic T cell
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
T_{FH}	Follicular T helper cell
TGF- β	Transforming growth factor-β
T_H	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumor necrosis factor receptor superfamily
T_{REG}	Regulatory T cell
UV	Ultraviolet
xg	Times gravity

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1. Introduction

1.1. Immune system

The main function of the immune system is to protect the body from pathogenic agents. It consists of a variety of organs, tissues, cells and molecules, which ensure this protection. The main tasks of the immune system are immunological recognition, immune effector functions, immune regulation and immunological memory.

The immune system can be classified into the innate and adaptive immune system, which play important roles in the defense against pathogens (Murphy et al., 2012).

1.1.1. Innate immune system

The innate immune system provides antigen-nonspecific first line defense against pathogens. Innate immune responses occur rapidly on exposure to a pathogen and are provided by a variety of components, such as physical and chemical barriers (skin and antimicrobial proteins of mucosa), the complement system and phagocytic cells, such as dendritic cells, macrophages, natural killer cells and granulocytes (neutrophils, eosinophils and basophils) as well as mast cells, which release enzymes and toxic proteins, when activated. These components ensure the functions of the innate immune system, namely acting as a barrier against pathogenic agents, their fast recognition, recruitment of immune cells to the sites of infection and activation of the adaptive immune system (Alberts et al., 2002).

Innate immune response initiates adaptive immune responses through antigen presentation. Thereby an important step is the maturation of dendritic cells. Immature dendritic cells are activated by recognition of common pathogenic structures via their toll-like receptors (TLR) and other pathogen recognition receptors. Activated dendritic cells act as antigen presenting cells (APC) and carry pathogenic antigens to peripheral lymphoid organs, where they present them to T cells. Besides antigen presentation, activated dendritic cells express specialized co-stimulatory molecules, which are essentially required for the activation of naive T cells and their differentiation into effector cells (Janeway and Medzhitov, 2002).

1.1.2. Adaptive immune system

Adaptive immune responses are initiated in peripheral lymphoid organs: lymph nodes, spleen and mucosa-associated lymphoid tissue (e.g. Peyer's patches). Key components of the adaptive immune system are lymphocytes. There are two types of lymphocytes: B and T cells, which have different roles in the immune system. They are distinguished from each other by the sites of maturation and expression of distinct types of antigen receptors (Murphy et al., 2012).

B cells develop in the bone marrow and further mature in the periphery. The specific antigen receptor of B cells is the B cell receptor (BCR), or surface immunoglobulin (sIg). After an antigen binds to the BCR, the B cell gets activated and differentiates into memory B cells or plasma cells. Plasma cells in their turn secrete soluble antibodies (immunoglobulins) and act in the humoral immune response (Higdon et al., 2016).

T cell precursors arise from a common lymphoid progenitor and enter the thymus, where they develop into naive T cells (Gasteiger and Rudensky, 2014). After they have completed their development in the thymus, naive T cells enter the bloodstream and circulate between blood and peripheral lymphoid tissues until they encounter their specific antigen. The antigen receptor of T cells is the T cell receptor (TCR). After first encounter to the antigen, naive T cells get activated and proliferate and differentiate into effector T cells. This process constitutes the primary immune response (Janeway et al., 2001). Effector cells have different functions (see 1.2.); however, their common feature is to respond quickly and efficiently as soon as they encounter their specific antigen on target cells. This response results in the removal of the foreign antigen from the organism (Davis and Bjorkman, 1988). T cells can be divided into three functional classes: CD8 cytotoxic T cells (T_C cells), which have a killing function, CD4 helper T cells (T_H cells), which have an activating function, and regulatory T cells (T_{REG} cells), which control immune responses by suppressing the activity of other lymphocytes (Murphy et al., 2012).

Unlike the innate immune system, the adaptive immune system is highly specific and can provide long-lasting protection by the creation of immunological memory.

1.1.2.1. Immunological memory

Immunological memory is one of the most important tasks of the adaptive immune system. It enables the immune system to respond more rapidly and effectively to previously encountered pathogens und thus protects the organism from diseases (Murphy et al., 2012). Memory immune cells should fulfill three main criteria. First, they should be long-lived and maintain independently of stimulation or antigen persistence. Second, they should be specific for a particular antigen. Third, memory cells should be intrinsically activated by the previously encountered antigen (Farber et al., 2016). After the clearance of an antigen, most of the effector T and B cells die; however, some of them survive and become long-lived memory cells (Murphy et al., 2012). These memory T and B cells fulfill criteria of memory immune cells; therefore, they can be considered as the bases of the immunological memory.

In this work, altered cytokine production was analyzed in memory CD4 T cells. Therefore, a short overview of the differentiation of naive T cells into memory T cells will be given.

There are two types of memory T cells: central memory and effector memory T cells (Figure 1). Central memory T cells (T_{CM}) express cell surface markers CCR7 and CD62L. These receptors are also expressed by naive T cells and are responsible for the migration of cells into T cell areas of secondary lymphoid organs. Upon restimulation, T_{CM} cells rapidly lose CCR7 and CD62L and differentiate into effector memory T cells. However, further differentiation into effector T cells is delayed and these effector T cells express few amounts of cytokines.

Effector memory T cells (T_{EM}) lack CCR7 and CD62L. They express inflammatory chemokines and can rapidly migrate into inflamed tissues. Early after restimulation, effector memory T cells rapidly mature into effector T cells and secrete large amounts of cytokines (Sallusto et al., 2004).

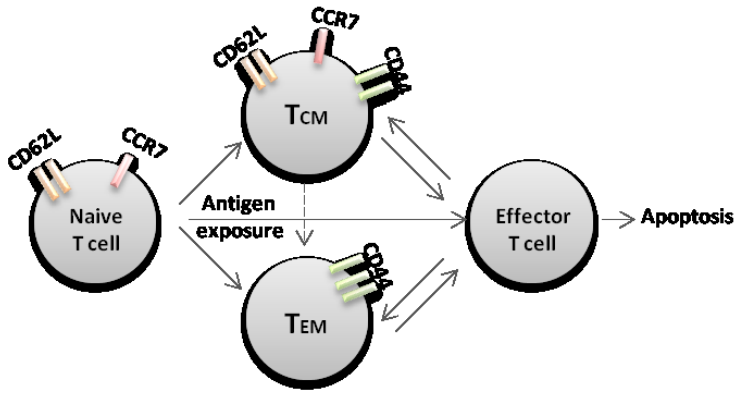

Figure 1. Differentiation of memory T cells.
Central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) arise from activated naive T cells or activated effector T cells. T_{CM} and T_{EM} cells are distinguished from each other by the expression of CCR7 and CD62L. After restimulation, they mature into effector T cells. T_{CM} cells can also differentiate into T_{EM} cells early after restimulation. After clearance of the antigen, most of the effector cells undergo apoptosis within a few days.

1.2. T cell activation and differentiation

Naive T cells differentiate into effector T cells after they get activated. Activation of naive T cells is initiated after they encounter their specific antigen, which is presented to them by antigen presenting cells (APCs) in following manner: APCs internalize pathogenic structures in peripheral tissues and degrade proteins into peptides. Antigenic peptides fulfill certain structural criteria that allow APCs to load these peptides onto special surface molecules, so called major histocompatibility complex (MHC) class I and II molecules. In this way, APCs present antigens to T cells in secondary lymphoid organs and initiate antigen-specific immune responses by effector T cells or immunological tolerance by regulatory T cells (Guermonprez et al., 2002).

Thus, a mature naive T cell gets activated, when it recognizes the appropriate peptide:MHC complex presented by APCs. However, complete differentiation of effector and regulatory T cells requires two more signals: These are on the one hand the interaction of co-stimulatory molecules (B7.1 and B7.2) on the surface of APCs with the co-stimulatory receptor on T cells CD28 and on the other hand cytokines, which determine the direction of T cell differentiation (Murphy et al., 2012).

Naive T cells have the ability to differentiate into several functional cell subtypes, which are specialized for certain immunological responses. For example, naive CD8 T cells differentiate

into cytotoxic effector CD8 T cells (T_C cells), which recognize peptide:MHC I complexes on target cells invaded by the pathogen and kill them. CD4 T helper cells (T_H cells) recognize peptide:MHC II complexes presented by APCs and initiate a pathogen-directed immunological response by activation of other cells of the immune system.

There are several subtypes of CD4 T cells, e.g. T_H1, T_H2, T_H17, T_{FH} and T_{REG} cells (Figure 2). Except for T_{REG} cells, which have an inhibitory function and limit the extent of the immune response, all other CD4 T cell subtypes have an activating function (Sallusto et al., 2004). T_H1 cells stimulate cells of the innate immune system (e.g. macrophages) to kill target cells invaded by intracellular bacteria and provide co-stimulatory signals for B cells to produce antibodies against extracellular pathogens. T_H2 cells are required for the class switching of B cells to produce IgE antibodies to fight extracellular parasite infections and are important for allergies. T_H17 cells stimulate neutrophils to clear extracellular bacteria and promote barrier integrity. T_{FH} cells provide help for B cells to produce antibodies in lymphoid follicles. T_{FH} cells can produce cytokines, characteristic for T_H1 and/or T_H2 cells. T_{REG} cells suppress effector T cell responses to limit the extent of immune responses and prevent autoimmunity (Caza and Landas, 2015).

The balance between T cell subtypes is important for the efficient functioning of the immune system (Caza and Landas, 2015). It is known that the predominance of T_H1 or T_H2 cell responses is often characteristic for the development of chronic autoimmune diseases or allergies, respectively (Murphy et al., 2012).

The functions of T cell subtypes are mainly defined by the production of mediator proteins, called cytokines (Caza and Landas, 2015).

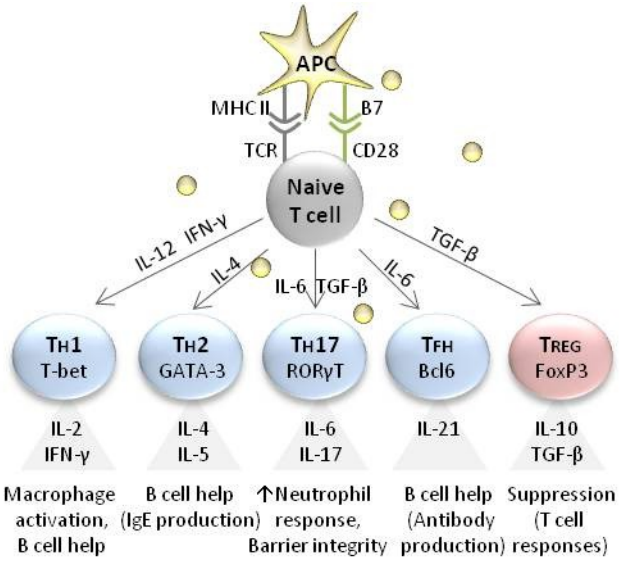

Figure 2. Differentiation of naive CD4 T cells.
Antigen producing cells (APC) provide activating signals to naive T cells via the T cell receptor (TCR) and the co-stimulatory receptor CD28. Activated T cells are able to differentiate into several T cell subtypes, e.g. T_H1, T_H2, T_H17 cells, follicular helper T cells (T_{FH}) or regulatory T cells (T_{REG}). The direction of differentiation is guided by cytokines released by APCs and other cells of the innate immune system in response to infection. Each subtype of T cells is characterized by the expression of a certain master transcription factor and the pattern of released cytokines.

1.3. Cytokines

Cytokines are small proteins, which have important functions in the signaling of the immune system. Main biological activities of cytokines include both cellular and humoral immune responses, induction of inflammatory responses, regulation of hematopoiesis, control of cellular proliferation and differentiation and induction of wound healing (Dinarello, 2007).

Cytokines are produced by a variety of cells usually in response to an activating stimulus and initiate their biological effects through the binding to specific receptors on the surface of target cells. Cytokines can act in an autocrine manner (affect the behavior of the cell that releases the cytokine), a paracrine manner (affect the behavior of neighbor cell) and an endocrine manner (affect the behavior of distant cell) (Firestein et al., 2012).

Cytokines and their receptors can be grouped by structure into different families: the hematopoietin family, the interferon (INF) family and the tumor necrosis factor (TNF) family (Figure 3). The hematopoietin receptor superfamily includes homodimeric (e.g. receptors for erythropoietin) and heterodimeric receptors with involving the common chain (e.g. receptors

for IL-3, IL-5, GM-CSF, IL-2 and IL-15). IFN superfamily receptors include heterodimeric receptors without the common chain (e.g. receptors for IFN-α, IFN-β, IFN-γ and IL-10). Most members of TNF receptor superfamily (e.g., TNFR1 and TNFR2, CD40, Fas, CD30, CD27) are transmembrane proteins and are found as homotrimers (Murphy et al., 2012).

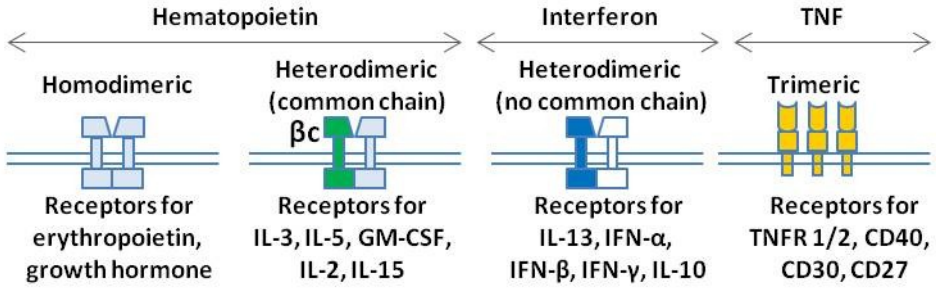

Figure 3. Cytokine receptor families.
The hematopoietin receptor family includes homo- and heterodimeric receptors with the common chain. The interferon (IFN) receptor family includes heterodimeric receptors without the common chain. The tumor necrosis factor (TNF) receptor family includes homotrimeric receptors.

Hematopoietin and IFN receptors signal through protein tyrosine kinases of the Janus kinase (JAK) family, thereby activating transcription factors known as Signal transducers and activators of transcription (STATs). Phosphorylated STAT molecules enter the nucleus and initiate the expression of selected genes (e.g. for growth and differentiation of lymphocyte subsets). Stimulation of TNF receptors are mediated either by Fas and tumor necrosis factor receptor 1 (TNFR1) or only TNFR2. TNFR1 contains a cytoplasmic death domain and is expressed by a wide range of cells. Dependent on the cell type, it induces apoptosis through procaspase activation or pro-inflammatory responses (such as production of TNF-α) through the activation of transcription factors, such as Nuclear factor of κB (NF-κB) and Activator protein 1 (AP-1) via TNF receptor-associated factor 2 (TRAF-2). In contrast to TNFR1, TNFR2 lacks a death domain and is expressed mostly on T cells. TNFR2 activates the NF-κB pathway, promoting cell survival rather than cell death (Murphy et al., 2012).

Inactivation of cytokine signaling occurs through negative feedback loops and involves dephosphorylation of cytokine receptor complexes, i.e. JAKs and STATs, by tyrosine phosphatases (Murphy et al., 2012).

Properties of two main cytokines, IFN-γ and IL-2, which were investigated in this work are given below.

1.3.1. Pleiotropic properties of IFN-γ

The interferon (IFN) family includes two main classes of interferons, type I IFNs (e.g., IFN-α, IFN-β) and type II IFN (only IFN-γ) (Platanias, 2005). As this work focuses mainly on IFN-γ, an overview of IFN-γ will be given.

IFN-γ was discovered about 50 years ago through its antiviral activities (Wheelock, 1965). The binding chain of IFN-γ receptor (IFN-γR) is located on human chromosome 6 (Farrar and Schreiber, 1993). IFN-γ can be produced by various cell types of the immune system, among them CD4 T cells, mainly T_H1 cells (Mosmann and Coffman, 1989; Sanders et al., 1988), CD8 T cells (Kasahara et al., 1983), B cells (Bao et al., 2014), natural killer cells (NK cells) (Boehm et al., 1997; Luetke-Eversloh et al., 2014), natural killer T cells (NKT cells) (Kronenberg, 2005) as well as macrophages and dendritic cells (Frucht et al., 2001).

Since IFN-γ plays a crucial role in immune responses, regulation of its expression is tightly controlled by multiple stimuli. IFN-γ production by T cells and NK cells is stimulated by IL-12 and IL-18 secreted by APCs (Okamura et al., 1998). Many transcription factors have been shown to contribute to the production of IFN-γ, among them the master transcription factor of T_H1 cells T-bet (Szabo et al., 2000) as well as STAT4 (Carter and Murphy, 1999), AP-1 (Barbulescu et al., 1998), Nuclear factor of activated T cells (NFAT) and NF-κB (Sica et al., 1997). In addition, IFN-γ expression is negatively regulated by signals of transforming growth factor β (TGF-β) (Gorelik and Flavell, 2000; Kulkarni et al., 1993), IL-6 (Diehl et al., 2000) and the transcription factor Suppressor of cytokine signaling 1 (SOCS1) (Alexander et al., 1999; Fujimoto et al., 2000).

IFN-γ participates in diverse immunological responses. As a major effector cytokine of T_H1 cells, IFN-γ production amplifies T_H1 cell-regulated responses by enhanced differentiation of naive CD4 T cells towards T_H1 cells in a positive feedback loop (Bradley et al., 1996; Das et al., 2001; Lighvani et al., 2001; Wakil et al., 1998; Zhang et al., 2001). Also, it has been reported that IFN-γ is able to stimulate the production of IL-12 by phagocytes (Yoshida et al., 1994) that may further drive T_H1 differentiation. In contrast, IFN-γ has been shown to interfere with other T_H cell responses, as it inhibits IL-4 secretion by T_H2 cells (Gajewski and Fitch, 1988; Gajewski et al.,

1988) as well as IL-17 secretion by T_H17 cells (Cruz et al., 2006; Harrington et al., 2005; Park et al., 2005).

Apart from its effects on T cell differentiation, IFN-γ plays an instrumental role in controlling intracellular bacterial infections via priming of innate immune cells. It increases antigen processing and presentation by upregulation of MHC class I and II antigen presenting pathways (Boehm et al., 1997). Furthermore, IFN-γ may enhance antibacterial activity of macrophages and thus plays a crucial role in the host defense against pathogens. Correspondingly, mice lacking IFN-γ or IFN-γR showed increased susceptibility to poorly virulent *Mycobacteria* species and certain parasites (Huang et al., 1993; Kamijo et al., 1993; van den Broek et al., 1995). Strikingly, complete or partial deficiency of IFN-γR in humans is strongly associated with susceptibility to *Mycobacteria* and *Salmonella*, early onset of infection and premature death. Moreover, impaired IFN-γ signaling leads to poor granuloma formation and uncontrolled growth of bacteria within macrophages (Doffinger et al., 2000; Jouanguy et al., 1999a; Jouanguy et al., 1999b). Individuals deficient for IFN-γ itself die very early from sepsis due to decreased neutrophil mobility and NK cell activity, demonstrating the immunomodulatory properties of IFN-γ (Davies et al., 1982).

There is increasing evidence for the relevance of IFN-γ in anti-tumor immunity. Initial hints have come from the fact that mice lacking IFN-γR developed tumors induced by chemical agents more rapidly and more frequently than wild-type mice (Kaplan et al., 1998). However, systemic administration of IFN-γ demonstrated limited efficacy in mice and humans with cancers. Further research showed that the therapeutic efficacy of IFN-γ is rather dependent on the responsiveness of tumor cells to IFN-γ. Anti-tumor effects of IFN-γ can be explained by its cytostatic, pro-apoptotic and anti-proliferative features (Tannenbaum and Hamilton, 2000).

Aside from its functions in host defense and anti-tumor immunity, IFN-γ has been shown to contribute to autoimmune diseases, such as autoimmune nephritis (Heremans et al., 1978), systemic lupus erythematosus (SLE) (Baechler et al., 2003; Lee et al., 2001), multiple sclerosis (Panitch et al., 1987) and diabetes mellitus (Sarvetnick et al., 1988). The mechanisms by which IFN-γ contributes to autoimmune diseases remain unclear. However, several experiments shed some light on the role of IFN-γ in autoimmunity. Thus, it has been shown that increased epidermal transgenic expression of IFN-γ leads to the development of glomerulonephritis through increased production of anti-dsDNA and anti-histone autoantibodies by B cells (Seery,

2000). This effect could be achieved due to the contribution of IFN-γ to antibody production and antibody class switching in B cells (Schroder et al., 2004). Also, IFN-γ might contribute to autoimmune diseases through the infiltration of target organs by IFN-γ producing T cells, causing end organ damage (Hu and Ivashkiv, 2009; Schwarting et al., 1998a; Schwarting et al., 1998b). CXCR3, a T_H1-associated chemokine receptor, has been found to be increased in lupus nephritis and was shown to contribute to tissue inflammation and autoantibody production (Lacotte et al., 2013).

Interestingly, in mouse models of collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) IFN-γ showed rather a protective function, limiting tissue damage associated with inflammation (Ferber et al., 1996; Manoury-Schwartz et al., 1997; Willenborg et al., 1996). The protective role of IFN-γ in these experimentally induced autoimmune conditions might be explained by the fact that IFN-γ on the one hand inhibits IL-17 production by pathogenic T_H17 cells and on the other hand enhances the activity of regulatory T cells (Kelchtermans et al., 2008).

Taken together, IFN-γ is involved in innate and adaptive immune responses. It plays a crucial role in protection of the organism against pathogens by increasing activity of macrophages and inducing T_H1 responses; it has immunomodulatory functions by enhancing the activity of NK cells, neutrophils and leucocytes, by inhibiting the activity of T_H17 cells and enhancing the activity of T_{REG} cells. IFN-γ has even anti-tumor functions by its direct cytotoxic effects on cancer cells and activation of the adaptive immune system against cancer cells (Lugade et al., 2008). However, IFN-γ contributes significantly to a variety of autoimmune diseases in a pro-inflammatory manner. The functions of IFN-γ are summarized in Figure 4.

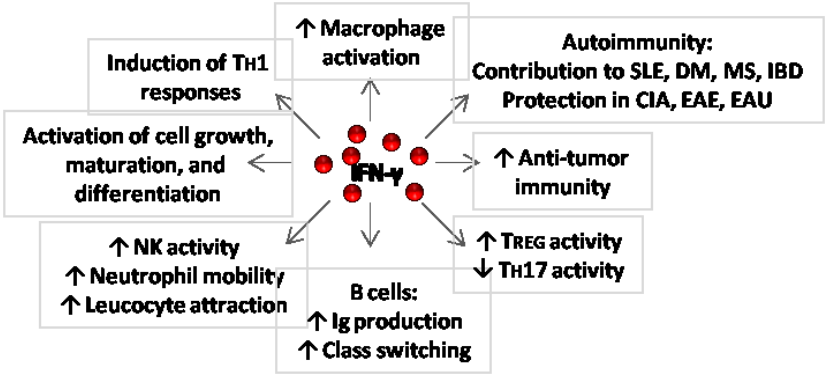

Figure 4. Functions of IFN-γ.
A schematic illustration. SLE - systemic lupus erythematosus, DM - diabetes mellitus, MS - multiple sclerosis, IBD - inflammatory bowel disease, CIA - collagen-induced arthritis, EAE - experimental autoimmune encephalomyelitis, EAU - experimental autoimmune uveitis.

1.3.2. Immunomodulatory and immunostimulatory properties of IL-2

The cytokine IL-2 is of central importance for immune responses (Fuhrmann et al., 2016). IL-2 was first discovered in the 1970ies due to its capability to induce T cell proliferation and differentiation (Gillis et al., 1978; Gillis and Smith, 1977; Morgan et al., 1976; Smith, 1988), that is why it was initially called T cell growth factor. IL-2 is a 15kDa cytokine, which can be produced by a variety of cells, among which predominantly by activated CD4 cells (Leonard, 2001; Setoguchi et al., 2005) and to a lesser extent by CD8 cells (Cheng et al., 2002; Paliard et al., 1988) as well as dendritic cells (Granucci et al., 2001), NKT cells (Yui et al., 2004) and mast cells (Hershko et al., 2011).

Nowadays, it is known that IL-2 can act not only as a T cell growth factor, but it has a large range of functions in the immune system. Besides induction of T cell proliferation, IL-2 also promotes immunoglobulin secretion and proliferation of B cells (Mingari et al., 1984), it induces proliferation of NK cells and enhances their cytolytic activity (Siegel et al., 1987). Strong IL-2 signals are required for the differentiation of both CD4 and CD8 T cells into effector cells. In contrast, weak IL-2 signals lead to the differentiation of CD4 T cells into T_{FH} cells or central memory T cells. IL-2 is required for CD8 T cells to survive and become long-lived memory CD8 T cells (Boyman and Sprent, 2012).

Apart its immunostimulatory functions, IL-2 has an essential role in immune tolerance and homeostasis (Klebb et al., 1996). First, IL-2 is able to promote activation-induced cell death

(AICD) of autoreactive immune cells (Kneitz et al., 1995; Lenardo et al., 1999). In addition, IL-2 is essential for the development and maintenance of $CD4^+FoxP3^+$ regulatory T cells (T_{REG} cells) (Malek and Castro, 2010). This has been demonstrated in experiments, where mice lacking IL-2 developed severe lymphoproliferative and autoimmune disorders (Sadlack et al., 1995; Sadlack et al., 1993; Schorle et al., 1991; Suzuki et al., 1995a). Here, the main reason for autoimmunity associated with IL-2 deficiency was a defect in T_{REG} cells. Regulatory T cells are generally known to inhibit proliferation and differentiation of self-reactive T cells (Sakaguchi, 2004; Shevach, 2009). IL-2 signaling is critically required for the proper functioning of T_{REG} cells, as IL-2 maintains stable expression of their master transcription factor, FoxP3, and therefore, lineage stability and functioning (Fontenot et al., 2005). Thus, in the absence of IL-2, the numbers of T_{REG} cells decline and the numbers of T_H17 cells increase, which leads to enhanced susceptibility to autoimmune diseases (D'Cruz and Klein, 2005; Fontenot et al., 2005; Littman and Rudensky, 2010).

Taken together, IL-2 is an important immunomodulatory cytokine, which has a wide spectrum of biological actions: On the one hand, driving T cell proliferation and differentiation, on the other hand, suppressing potentially harmful autoimmune reactions. Immunomodulatory and immunostimulatory functions of IL-2 are summarized in Figure 5.

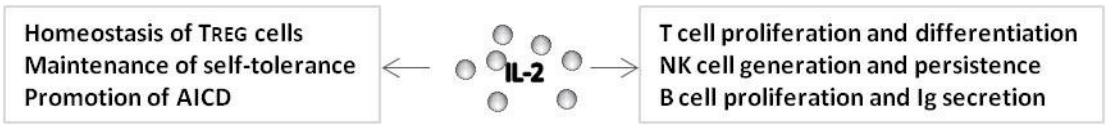

Figure 5. Functions of IL-2.
A schematic illustration. AICD – activation-induced cell death.

Since IL-2 is a crucial cytokine for the immune system, its signaling and the regulation of its expression represent an important topic. IL-2 expression in T cells is induced after stimulation of both the T cell receptor (TCR) and the co-stimulatory receptor CD28, which leads to the activation of signaling cascades, resulting in the activation and migration of several transcription factors into the nucleus, where they bind to the IL-2 promotor and induce IL-2 expression. Main transcription factors involved in IL-2 production are NFAT, AP-1, including c-Fos and c-Jun, NF-κB and the Octamer transcription factor 1 (OCT-1) (Bendfeldt et al., 2012).

In turn, IL-2 expression is inhibited by cAMP responsive element modulator (CREM) (Barton et al., 1996; Juang et al., 2005), Zinc finger E-box (ZEB) (Yasui et al., 1998), T-box transcription factor (T-bet) (Hwang et al., 2005) and by an autoregulatory feedback loop, which depends on the activation of STAT5 (Villarino et al., 2007) and B lymphocyte-induced maturation protein 1 (BLIMP-1) (Gong and Malek, 2007).

Once IL-2 is produced, it acts through the binding to its specific receptor. The IL-2 receptor (IL-2R) consists of three subunits: α- (IL-2Rα, CD25), β- (IL-2Rβ, CD122) and the common receptor γ-chain (γc, CD132). β- and γc-subunits form an intermediate-affinity binding to IL-2, whereas together with the α-subunit (CD25) they form a high-affinity IL-2R complex (CD132, CD122 and CD25) (Cacalano and Johnston, 1999). A very similar structure to IL-2R has the receptor for IL-15, namely they share their β- and γc-chains (Waldmann, 2006). IL-2-IL-2R binding results in signal transduction through the activation of three major pathways: (1) JAK/STAT-, (2) Mitogen-activated protein kinase (MAPK-) and (3) Phospatidylinositol 3 kinase (PI3K-) dependent signaling pathways, which basically mediate proliferation and survival signaling (Benczik and Gaffen, 2004).

Being an important cytokine with pleiotropic functions, IL-2 administration or its blockade has been approved for therapeutic approaches for a range of diseases. However, the modulation of IL-2 response is strongly context-dependent. For example, enhancement of its effector activities, such as expansion and activation of cytotoxic T cells and NK cells may be beneficial for the elimination of cancer cells (Rosenberg, 2012; Schwartzentruber et al., 2011), whereas blockade of its effector responses (Borie et al., 2003; Changelian et al., 2003; Kremer et al., 2009) or enhancement of the activity of regulatory T cells (Humrich and Riemekasten, 2016) may be beneficial for fighting autoimmunity and inflammation.

1.4. Systemic lupus erythematosus

1.4.1. Definition, etiology and epidemiology of SLE

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease which is characterized by autoantibody production and infiltration of target organs with inflammatory cells (Moulton and Tsokos, 2011). SLE can affect almost every organ system of the body and therefore, it has a very heterogeneous clinical manifestation, which may delay the early diagnosis (Hiepe, 2014). A

specific cause of SLE is still unknown, however, genetic, environmental and hormonal factors as well as several drugs have been discussed to trigger lupus (Alexander et al., 2015).

The frequency of SLE varies worldwide by race and ethnicity (Danchenko et al., 2006). In Germany, the prevalence of SLE was estimated in 2002 to be 36.7 per 100,000 with a female to male ratio of 4:1 (Brinks et al., 2014). SLE frequently starts in women of childbearing age (Aringer and Hiepe, 2011), but it can also affect men (Brinks et al., 2016) and children at the age of up to 19 years (Pineles et al., 2011).

Early diagnosis and advances in general medical care along with a better understanding of the pathogenesis of the disease contributed to an increase of 10-year survival rate from 5% in 1955 to 95% in 2003 (Cervera et al., 2003; Chehab et al., 2011). The main cause of the mortality in early SLE is on the one side the active disease itself with multiple organ damage and on the other side infectious complications, related to immunosuppressive treatment (Cervera et al., 2003). The causes of the late mortality are long-term side effects of corticosteroids and other drugs, including atherosclerosis and hypertension with their consequences as well as direct tissue damage of these drugs (Manzi et al., 1997; Salmon and Roman, 2001).

1.4.2. Cytokines in the pathogenesis of SLE

Various aspects of the immune system are involved in the pathogenesis of SLE, demonstrating the complexity of the disease (Mok and Lau, 2003). In particular, SLE is characterized by increased apoptosis of a variety of cells. In its turn, the clearance of apoptotic cells is impaired and results in the accumulation of cellular debris and increased presentation of autoantigens from these apoptotic cells to T cells (Alexander et al., 2015). Activated autoreactive T cells start to provide activating signals to B cells, which in their turn begin to produce autoantibodies and differentiate into autoantibody-producing memory plasma cells. These autoantibodies are predominantly directed against nuclear structures and RNA-binding proteins. Direct action of these autoantibodies on cells and/or formation of immune complexes results in further tissue damage (Hiepe and Radbruch, 2016; Winter et al., 2015). Figure 6 shows a simplified scheme of SLE pathogenesis.

There is increasing evidence that cytokines play an important role in the pathogenesis of SLE. It is known that activation of dendritic cells by immune complexes results in increased production

of IFN-α (Lovgren et al., 2004). In its turn, IFN-α was shown to promote apoptosis (Strandberg et al., 2008) and enhance antibody production by B cells (Baccala et al., 2007; Le Bon et al., 2006). IFN-α seems to play an important role in SLE, since its increased serum levels were shown to positively correlate with serological and clinical signs of the disease (Bauer et al., 2006; Dall'era et al., 2005; Ytterberg and Schnitzer, 1982). Moreover, gene expression analyses demonstrated so called IFN-α signature (genetic association with IFN-α-related pathways) in the majority of SLE patients (Baechler et al., 2003; Bezalel et al., 2014; Feng et al., 2006). Besides IFN-α, production of many other cytokines, such as interleukins, TNF-α and IFN-γ was shown to be altered in SLE (Apostolidis et al., 2011; Lauwerys and Houssiau, 1998; Ohl and Tenbrock, 2011; Yap and Lai, 2010).

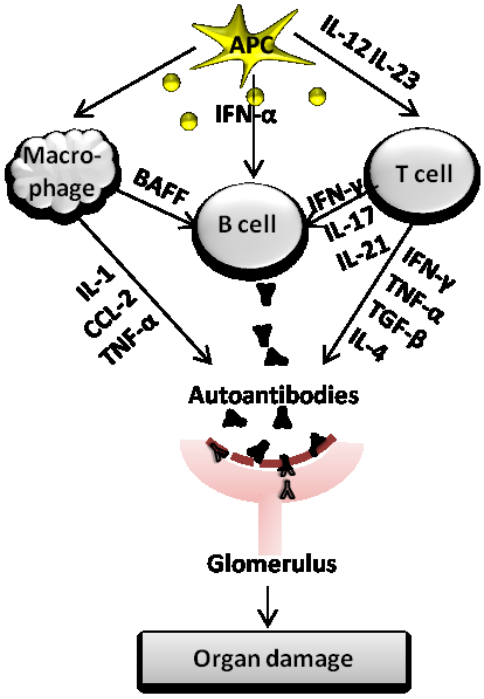

Figure 6. SLE pathogenesis.
A simplified visualization. Increased production of IFN-α by antigen presenting cells (APC) initiates a cascade of events that results in the activation of autoreactive B and T cells. Autoreactive B cells produce antibodies, which can form immune complexes with structures released from apoptotic cells. These immune complexes deposit in target organs, causing tissue damage. On the one hand, T cells provide help to autoreactive B cells to produce autoantibodies; on the other hand, they infiltrate target organs, causing local inflammation. Both processes are mediated by cytokines and chemokines, which may induce apoptosis and necrosis of target tissues, resulting in fibrosis and end organ damage.

Altered expression of several cytokines has been a subject of intensive research during the last decades. However, the roles of many cytokines are not completely understood yet. Table 1 summarizes known alterations of several important cytokines in SLE.

Table 1. Altered cytokine expression during SLE.

Cytokine	Altered expression during SLE
IFN-α	- High serum levels in SLE patients correlated with disease activity (Bengtsson et al., 2000). - Half of SLE patients were characterized by dysregulated gene expression of IFN-α pathway (Baechler et al., 2003).
IFN-γ	- Increased expression of IFN-γ correlated with disease activity in NZBxW lupus-prone mice. IFN-γ administration accelerated lupus and its blockade delayed the onset of the disease in these mice (Jacob et al., 1987). - Early IFN-γ administration in MRL/lpr lupus mice protected from SLE and its late administration accelerated disease activity (Nicoletti et al., 2000). - In SLE patients both high (Uhm et al., 2003) and low (Min et al., 2001) serum levels of IFN-γ were shown to correlate with disease activity.
IL-1	- Increased IL-1 expression in kidneys of diseased MRL/lpr and NZBxW lupus-prone mice correlated with disease activity. Low does administration of IL-1 accelerated nephritis in these animals (Brennan et al., 1989). - IL-1 expression was increased in patients with lupus nephritis (Takemura et al., 1994). - Enhanced production of IL-1 by monocytes correlated with disease activity in SLE patients (Suzuki et al., 1995b). - Elevated IL-1 levels were characteristic for flares in patients with extra-renal manifestations, but not for kidney involvement in SLE (Sturfelt et al., 1997).
IL-10	- Treatment of NZBxW lupus-prone mice with IL-10 accelerated disease activity and its blockade delayed the onset of nephritis in these mice (Ishida et al., 1994). - IL-10 deletion in young MRL/lpr lupus mice resulted in exacerbation of the disease, showing its protective role in early disease (Yin et al., 2002). - In SLE patients, increased IL-10 serum levels correlated with disease activity (Houssiau et al., 1995; Llorente et al., 1995).
IL-17	- Increased IL-17 production in mice and patients with SLE correlated with disease activity (Nalbandian et al., 2009).
IL-2	- IL-2 production by T cells was decreased in mice (Altman et al., 1981) and humans with active SLE (Alcocer-Varela and Alarcón-Segovia, 1982; Linker-Israeli et al., 1983). - IL-2 administration in mice (Humrich et al., 2010) and humans with SLE seemed to be beneficial for SLE treatment (von Spee-Mayer et al., 2016).
IL-21	- Increased expression of IL-21 correlated with lupus activity in humans and mice with SLE (Bubier et al., 2009; Nakou et al., 2013; Terrier et al., 2012).
IL-6	- IL-6 blockade ameliorated SLE signs in MRL/lpr and NZBxW lupus-prone mice (Finck et al., 1994a; Kiberd, 1993). - Increased IL-6 serum levels correlated with disease activity in SLE patients (Stuart et al., 1995).

TNF-α	- TNF-α expression was decreased in diseased NZBxW lupus-prone mice (Jacob and McDevitt, 1988). - Therapeutic administration of TNF-α improved survival of NZBxW lupus-prone mice (Gordon et al., 1989). - Increased TNF-α expression correlated with disease activity in MRL/lpr lupus mice (Boswell et al., 1988; Yokoyama et al., 1995). - Blockade of TNF-α in patients with rheumatoid arthritis, ankylosing spondylitis or Crohn's disease may lead to lupus-like symptoms (Carlson and Rothfield, 2003; De Rycke et al., 2003; Shakoor et al., 2002). - Increased TNF-α levels correlated with disease activity in a small group of SLE patients (Aderka et al., 1993; Aringer et al., 2002; Gabay et al., 1997; Maury and Teppo, 1989). - In selected SLE patients with nephritis, TNF-α blockade might be a useful therapeutic option (Aringer et al., 2004; Hayat et al., 2007).

Whereas the production of many pro-inflammatory cytokines is increased in SLE, the production of IL-2, a crucial cytokine for the regulation of immune tolerance and homeostasis (de la Rosa et al., 2004), was shown to be decreased. Another remarkable point is the heterogeneous production of IFN-γ in SLE. As a major pro-inflammatory cytokine of the T_H1 cells, IFN-γ might play a certain role in the pathogenesis of SLE; however, this hypothesis has not been proved yet. In order to more precisely investigate IL-2 and IFN-γ alterations in SLE, the features of IL-2 and IFN-γ producing cells were analyzed in this work. Further a short introduction into the roles of these two cytokines in SLE will be given.

1.4.2.1. The role of IFN-γ in SLE

IFN-γ expression by T cells of NZBxW lupus-prone mice (NZBxW is a spontaneous model of SLE (Perry et al., 2011); see 1.5.) was reported to be increased and correlated with the age of mice and the development of the disease (Enghard et al., 2006). Both early and recent studies showed that the treatment of NZBxW lupus-prone mice with IFN-γ resulted in the acceleration of the development of glomerulonephritis, in contrast, the treatment of these animals with anti-IFN-γ antibodies diminished severity of SLE, demonstrating the importance of IFN-γ in this model of lupus nephritis (Jacob et al., 1987; Schmidt et al., 2015).

Interestingly, the role of IFN-γ was shown to be heterogeneous in another (MRL/lpr) strain of lupus mice. MRL/lpr mice have an autoimmune MRL background and carry the *lpr* mutation within the *Fas* gene (Adachi et al., 1993), which results in lymphocyte proliferation and

increased immune complex formation, i.e. two characteristic features of SLE (Merino et al., 1993). MRL/lpr lupus mice lacking the IFN-γR exhibited reduced deposition of immune complexes and complement component 3 (C3) in glomerular capillaries and no proteinuria, demonstrating that glomerulonephritis in these mice was primarily mediated by IFN-γ producing cells (Haas et al., 1997). These observations were confirmed by Schmidt et al., as they showed that IFN-γ producing CD4 T cells infiltrated kidneys of both MRL/lpr and NZBxW lupus-prone mice and their production continuously increased with disease progression (Schmidt et al., 2015). However, Nicoletti et al. reported about a bidirectional role of IFN-γ in SLE. In detail, when IFN-γ was administered before onset of the disease it favorably modulated the signs of SLE and lowered the mortality of MRL/lpr lupus mice. By contrast, administration of IFN-γ after onset of the disease worsened the course of SLE in these animals, demonstrating both protective and pro-inflammatory functions of IFN-γ in the MRL/lpr mouse model of SLE (Nicoletti et al., 2000).

Clinical studies regarding IFN-γ expression in humans with SLE delivered conflicting results, as well. While some studies reported about increased IFN-γ levels in the sera of SLE patients and its correlation with disease activity (al-Janadi et al., 1993; Masutani et al., 2001; Tokano et al., 1999; Uhm et al., 2003), other studies reported about decreased IFN-γ levels in patients with active SLE (Horwitz et al., 1998; Min, 2001). These controversial observations regarding the role of IFN-γ in SLE manifestation both in humans and mice indicate a complex role of this cytokine in the regulation of systemic autoimmunity, such as SLE. Due to this inconsistency, it is of great importance to further investigate the role of IFN-γ in SLE.

1.4.2.2. The role of IL-2 in SLE

Early studies reported about a significant decrease in the production of IL-2 by T cells both in murine lupus models (Altman et al., 1981) and humans with SLE (Alcocer-Varela and Alarcón-Segovia, 1982; Linker-Israeli et al., 1983). Loss of IL-2 was associated with reduced numbers of T_{REG} cells. Since IL-2 has a crucial role for the maintenance of T_{REG} homeostasis, its defect is associated with the breakdown of immunomodulatory T_{REG} functions and the development of autoimmunity (Kammer, 2005). Recent studies demonstrated that IL-2-based immunotherapy led to the amelioration of SLE in both preclinical (Humrich et al., 2010) and clinical cases (von Spee-Mayer et al., 2016) and resulted in the reestablishment of the balance between effector

and regulatory T cells, demonstrating that IL-2 deficiency affects T_{REG} homeostasis in SLE and thus contributes to the pathogenesis of SLE.

1.4.3. Clinical manifestation and diagnosis of SLE

Due to the diversity of the pathogenesis of SLE, its clinical manifestations are also diverse. The majority of patients with SLE report about fever, fatigue, weight changes as well as lymphadenopathy, polyarthritis and myositis. Many patients display cutaneous manifestations (Figure 7), such as malar rash (so called butterfly rash over the nasal bridge and cheeks), photosensitivity and discoid rash mostly on sun-exposed areas (red plaques with plugging and scarring). In addition, alopecia, Raynaud phenomenon, vasculitis purpura and ulcera are frequently observed (Cojocaru et al., 2011).

Figure 7. Clinical manifestations of SLE.
(A) Skin lesions on the back and arms, (B) arthritis of hand joints (so called Jaccoud's arthropathy without radiological erosions), (C) discoid erythema on the right cheek and ear with keratosis and comedo-like lesions, (D) discoid erythema on the scalp with hyperkeratosis and alopecia (from (Kuhn et al., 2015)).

Further manifestations of SLE include polyserositis (pleuritis, pericarditis, etc.) with increased risk of cardiovascular diseases, pneumonitis, myocarditis and endocarditis (so called nonbacterial Libman-Sacks endocarditis). Over 40% of SLE patients develop lupus nephritis

(immune complex-mediated glomerulonephritis) and neurological changes. Direct autoantibody effects on cells with formation of blood clots or lysis of blood cells might result in antiphospholipid antibody syndrome or Coombs positive hemolytic anemia, respectively (Aringer and Hiepe, 2011; Herrmann et al., 2000; Kuhn et al., 2015).

Laboratory parameters of SLE include hematologic abnormalities, like leucopenia, lymphopenia and thrombocytopenia, as well as increased parameters of inflammation. Characteristic serological abnormalities for SLE are high levels of anti-nuclear autoantibodies (ANA), anti-dsDNA antibodies and low complement levels (Kuhn et al., 2015). Additional serological parameters are increased levels of anti-SM, anti-Ro, anti-C1q antibodies as well as identification of antiphospholipid antibodies and circulating immune complexes (Herlod, 2016).

In order to register the activity of SLE, several scoring systems have been generated, which include key clinical and laboratory parameters for SLE. The most common scoring system in use is the SLE Disease Activity Index (SLEDAI). In addition to disease activity, organ damage during SLE can be evaluated by damage index scoring, e.g. SLICC/ACR (Systemic Lupus International Collaborating Clinics/American College of Rheumatology). These scoring systems are important for standardized evaluation of SLE activity and organ damage and therapy decision making (Kuhn et al., 2015; Lam and Petri, 2005).

1.4.4. Treatment options for SLE

Application of medication in SLE is dependent on the disease severity and visceral manifestations with the main purpose to prevent irreversible organ damage. Treatment of SLE is mostly based on four groups of medications: non-steroidal anti-inflammatory drugs (NSAID) (Paracetamol, Nimesulid, Ibuprofen, Diclofenac); antimalarials (Hydroxychloroquine, Chloroquine); corticosteroids (Prednisolone) and immunosuppressants (Azathioprine, Methotrexate) (www.dgrh.de). Administration of these drugs is associated with increased complications, such as severe infections, gastrointestinal bleeding or perforation, osteoporosis, malignancies, increased atherosclerosis with severe cardiac complications, including myocardial infarction, representing a serious risk for patients' lives (Aringer and Hiepe, 2011; Bertsias et al., 2010; Cervera et al., 2003; Kuhn et al., 2015; Manzi et al., 1997; Salmon and Roman, 2001).

For patients with severe refractory SLE, further treatment options, such as autologous stem cell transplantation (ASCT), immune adsorption and plasmapheresis were suggested. With the help of immune adsorption and plasmapheresis autoantibodies can be directly removed from the blood (Stummvoll et al., 2009). During ASCT the whole immunological memory is depleted, both pathological and protective components, resulting in complete disappearance of autoantibodies, particularly anti-dsDNA antibodies (Alexander et al., 2009). Although these are highly risk-bearing therapy options, there are many reports about a long-term remission (Aringer and Hiepe, 2011).

Another field of SLE treatment is anti-cytokine therapies.

1.4.4.1. Anti-cytokine therapies for SLE

Since cytokines are involved in the pathogenesis of many autoimmune diseases, anti-cytokine therapies have proved to be beneficial, since they can dampen damaging inflammatory responses of autoreactive immune cells. Patients with unresponsiveness or incompatibility to conventional therapies could profit from anti-cytokine therapies. Particularly for SLE, the blockade of various cytokines has been studied in preclinical models and some of them have been tested in clinical trials, as well (La Cava, 2010).

IFN-γ blockade in NZBxW lupus-prone mice led to the amelioration of SLE activity (Jacob et al., 1987). Treatment of SLE patients with AMG 811, a human monoclonal anti-IFN-γ antibody, led to inhibition of INF-γ signaling; furthermore, it led to decrease of serum CXCL10 levels, which are known to be associated with SLE activity (Welcher et al., 2015). However, administration of AMG 811 did not improve clinical or serological signs in patients with discoid lupus erythematosus (Werth et al., 2017) and lupus nephritis (Boedigheimer et al., 2017).

IFN-α was shown to be one of the major cytokines, involved in the pathogenesis of SLE. Blockade of IFN-α with Sifalimumab proved to be a promising treatment option for SLE patients refractory to conventional therapies (Greth et al., 2017; Khamashta et al., 2016). However, treatment of SLE patients with Rontalizumab, another anti-IFN-α monoclonal antibody, failed to show efficacy (Kalunian et al., 2016).

TNF-α blockade was shown to be successful in patients with rheumatoid arthritis, ankylosing spondylitis and Crohn's disease; however, it can induce lupus-like symptoms in these patients, which disappear by termination of TNF-α blockade (Swale et al., 2003). In SLE particularly, anti-TNF-α therapy is not promising, but it might be useful for the treatment of polyarthritis, polyserositis and nephritis (Aringer et al., 2007).

IL-10 blockade in NZBxW lupus-prone mice reduced anti-dsDNA antibody titers, which serve as an indicator of SLE activity, and delayed the onset of glomerulonephritis (Ishida et al., 1994). Treatment of SLE patients with a murine monoclonal anti-IL-10 antibody (B-N10) resulted in improvement of cutaneous lesions and joint symptoms. However, all patients developed antibodies against murine B-N10 (Llorente et al., 2000).

Up to now, many of these medications are not admitted for SLE treatment. Only Belimumab, a fully human monoclonal antibody that inhibits the activity of B cells through the binding to B lymphocyte stimulator (BLyS) has recently been approved for the use in active SLE in Germany and shows promising results for treatment of lupus nephritis (Frieri et al., 2015).

Several pragmatic approaches for treatment of SLE are summarized in Figure 8 (Xiong and Lahita, 2014).

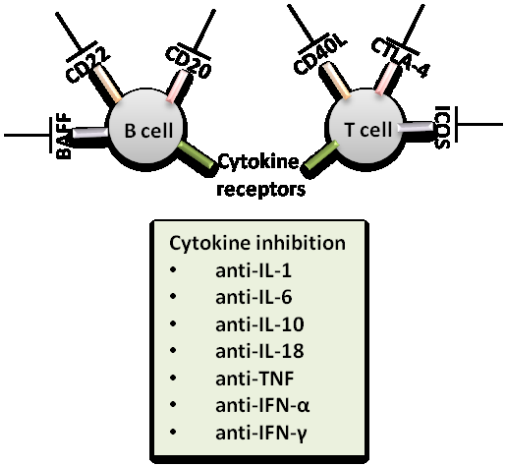

Figure 8. Targets for SLE treatment.
Inhibition of co-stimulatory and co-inhibitory receptors on B and T cells as well as blockade of survival factors and cytokine receptors might provide targets for SLE treatment. These mechanisms are nowadays under intensive research. BAFF – B cell activating factor, CTLA-4 – cytotoxic T lymphocyte-associated antigen 4, ICOS – inducible T cell co-stimulator (adopted from (Xiong and Lahita, 2014)).

Despite many therapy options, healing of SLE is impossible. The main goal of SLE therapies is to reach a remission and to prevent (further) organ damage with the minimum possible dose of glucocorticoids in order to avoid their toxicity (Kuhn et al., 2015). Therefore, there is still a need for more effective therapeutic approaches, which would specifically target pathogenic agents in SLE.

1.5. NZBxW murine model of SLE

NZBxW is a spontaneous mouse model of SLE, generated by the F1 hybrid of NZB (New Zeeland Black) and NZW (New Zeeland White) strains. Female NZBxW F1 hybrids display lupus-like phenotypes similar to those of SLE patients. Clinical manifestations of lupus in these mice include generalized swelling of lymph nodes, spleen enlargement, polyserositis and anasarca, which result in (often rapid) weight gain. Furthermore, these mice are characterized by elevated serum levels of anti-nuclear autoantibodies (ANA) and anti-dsDNA antibodies as well as proteinuria, as a result of immune complex-mediated glomerulonephritis. Disease progression in NZBxW lupus-prone mice correlates with their age. Clinical signs become apparent at the age of 5-6 months. Progressive immune complex accumulation in kidneys leads to terminal kidney failure and death at the age of 10-12 months. Unlike SLE patients or several other SLE mouse models (e.g. MRL/lpr and BXSB/Yaa), NZBxW mice do not produce autoantibodies against RNA-containing complexes (Perry et al., 2011; Theofilopoulos and Dixon, 1985).

Historically NZBxW lupus-prone mice have provided large contribution to our understanding of pathogenic mechanisms in SLE. Even nowadays NZBxW serves as a well suitable mouse model for research in this field.

1.6. Aims of this work

Systemic lupus erythematosus (SLE) is a complex autoimmune disease with multiple alterations of the immune system. Both cellular and humoral immune responses contribute to the disease pathogenesis (Alexander et al., 2015). Aberrant cytokine production by T cells may affect both innate and humoral immune responses in SLE, thus, representing an important potential target for drug intervention (Ohl and Tenbrock, 2011). Previous research has addressed the role of IL-2 and IFN-γ in SLE. In particular, decreased IL-2 expression has been reported in SLE and IL-2-based immunotherapy led to amelioration of the disease (von Spee-Mayer et al., 2016). In contrast to the protective functions of IL-2 in SLE, the role of another T cell-derived cytokine, IFN-γ, remains controversial. Some reports highlighted its beneficial effects during the early stage of the disease, whereas others suggested deleterious impact of IFN-γ in this autoimmune disease (Ohl and Tenbrock, 2011).

Based on this, the hypothesis of this work was that memory CD4 T cells that produce only IL-2 (IFN-γ^{-}IL-2^{+}) or only IFN-γ (IFN-γ^{+}IL-2^{-}) or both cytokines simultaneously (IFN-γ^{+}IL-2^{+}) may have distinct contributions to SLE pathogenesis. Therefore, the main aim of this work was to dissect the potential role of distinct cytokine producing memory CD4 T cell subsets in SLE. To achieve this, these cell subsets were analyzed in NZBxW lupus-prone mice. Cells were sorted using cytokine secretion assays, which allowed for extraction of living cytokine producing cells for subsequent determination of their gene expression profiles, proliferation capacities and apoptosis rates with an aim to define characteristic features and specific markers.

2. Materials and methods

2.1. Materials

2.1.1. Chemical products

Table 2. List of chemical products

Name	Manufacturer
Bovine serum albumin (BSA)	New England Biolabs
β-Mercaptoethanol	Thermo Fisher
Brefeldin A	Sigma-Aldrich
Carboxyfluorescein N-succinimidyl ester (CFSE)	eBioscience
Collagenase D	Roche Diagnostics GmbH
Collagenase from *Clostridium histolyticum*	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
DNAse I	Sigma-Aldrich
Ethanol	Carl Roth
Ethylenediaminetetraacetic acid (EDTA)	Merck
Fetal calf serum (FCS)	Merck
Formaldehyde	Merck
Glycerol	Carl Roth
Glycine	Carl Roth
Heparin	Biochrom AG
Ionomycin	Merck
Isoflorane	Baxter Deutschland GmbH
L-glutamate	Sigma-Aldrich
Mouse Pancoll	PAN-Biotech
Penicillin	Life Technologies
Percoll	VWR International GmbH
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich
Propidium Iodide (PI)	eBioscience
RPMI1640	Thermo Fisher
Saponin	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich

2.1.2. Antibodies

Table 3. List of fluorochrome-labeled antibodies for flow cytometric analysis

Antibody	Clone	Conjugate	Manufacturer
CD152 (CTLA-4)	UC10-4B9	Brilliant Violet 421™	BioLegend
CD223 (LAG-3)	eBioC9B7W	PerCP-eFluor® 710	eBioscience
CD25	pC61.5	APC	DRFZ
CD4	GK1.5	FITC, PE	DRFZ
CD44	IM7	Pacific Blue	DRFZ
CD62L	MEL-14	PE-Cy7	BD Biosciences
Granzyme B	GB11	FITC	BioLegend
IFN-γ	XMG1.2	PE-Cy7, APC	BD Pharmingen
IL-10	JES5-16E3	APC	eBioscience
IL-2	JES6-5H4	APC, PE	eBioscience

TNF-α	MP6-XT22	PerCP-Cy5.5	BioLegend
Ki-67	16A8	PE	BioLegend

Table 4. List of unlabeled antibodies and beads

Name	Clone	Manufacturer
B220	RA3.6B2	DRFZ
CD28	31.57	DRFZ
CD3	KT3	DRFZ
CD8	53-6.72	DRFZ
Fc-γR	2.4G2/75	DRFZ
Mac-1	M1170.15.11	DRFZ
Goat anti-Rat IgG Microbeads		Miltenyi Biotec

2.1.3. Kits and arrays

Table 5. List of kits and arrays

Name	Manufacturer
Ambion WT Expression Kit	Applied Biosystem GmbH
Annexin V Apoptosis Detection Set PE-Cyanine7	eBioscience
GeneChip® Hybridization, Wash, and Stain Kit	Affymetrix
GeneChip® Mouse Gene 2.0 ST Array	Affymetrix
GeneChip® WT Terminal Labeling and Controls Kit	Affymetrix
Mouse IFN-γ Secretion Assay Detection Kit in APC	Miltenyi Biotec
Mouse IL-2 Secretion Assay Detection Kit in PE	Miltenyi Biotec
Pacific Orange LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit	Invitrogen
Qiagen SYBRGreen Kit	QIAGEN
Reverse Transcriptase Superscript II-Kit	Life Technologies
RNeasy Plus Mini Kit	QIAGEN

2.1.4. Analysis software

Table 6. List of analysis software

Name	Manufacturer
FlowJo 7.6.5	FLOWJO, LLC
Graph Pad Prism 5	Graph Pad Software
R software	Open source

2.1.5. Consumables and devises

Table 7. List of consumables and devises

Name	Manufacturer
Bioanalyzer	Agilent Technologies
Cell strainer	BD
FACSAria cell sorter	BD Biosciences
FACSAria II cell sorter	BD Biosciences

Fortessa cell analyzer	BD Biosciences
Gene Chip-Array-Plattform	Affymetrix
Incubators	Thermo Scientific
Light microscope	Carl Zeiss
LS MACS columns	Miltenyi Biotec
LSRII cell analyzer	BD Biosciences
MACS separator	Miltenyi Biotec
Master cycler® personal	Eppendorf
MS2 minishaker	IKA®
NanoDrop ND-1000 Spectrophotometer	NanoDrop Technologies
Neubauer cell chamber	Paul Marienfeld GmbH & Ko. KG
Stratagene Mx3005P	Agilent Technologies
Multistix® 10 SG	Siemens
PMR-30 shaker	Grant Bio
Pre-separation filter (30 µm)	Miltenyi Biotec
Sterile benches	Heraeus

2.1.6. Recombinant proteins for cell culture

Table 8. List of cytokines for cell culture

Name	Manufacturer
IFN-γ	R&D SYSTEMS
IL-2	R&D SYSTEMS
IL-7b	R&D SYSTEMS

2.1.7. Buffers and media

Table 9. List of buffers and media

Name	Components
Cell culture medium	90% RPMI1640 10% FCS 10 mM L-glutamate 10 µg/ml β-mercaptoethanol 100 u/ml Penicillin
PBS	137 mM NaCl 2.7 mM KCL 1.4 mM KH_2PO_4 4.3 mM Na_2HPO_4 pH 7.4
PBS/BSA	PBS 0.5% BSA (w/v) pH 7.4
RBC lysis buffer	155 mM NH_4Cl 10 mM KCO_3 0.1 mM EDTA pH 7.3

2.1.8. Oligonucleotides

Table 10. List of oligodeoxynucleotides for qRT-PCR

Name	Sequence
Ctla4_fw	CCCTTCTgCAgTggTACCTTT
Ctla4_rv	TgACATAAATCTgCgTCCCgT
Dapl1_fw	ACAAgAgATgggCgTTTTggA
Dapl1_rv	gCTTTTgATgTgCCgTgTgAA
Gzmb_fw	CATgAAgTCAAgCCCCACTCT
Gzmb_rv	gTTCTTTgATgTTgTgggCCC
Il10_fw	TgAATTCCCTgggTgAgAAgC
Il10_rv	ATggCCTTgTAgACACCTTgg
Irf1_fw	ATAACTCCAgCACTgTCACCg
Irf1_rv	TTCCCTTCCTCATCCTCgTCT
Lag3_fw	ggggACTTCTCTCTgTggTT
Lag3_rv	ggACCCAATCAgACAgCTTgA
RPS18_fw	AggATgTgAAggATgggAg
RPS18_rv	TTggATACACCCACAgTTCg
Satb1_fw	ggCAACTCAggggAAAgAACA
Satb1_rv	ATgAgATggCCCgAgTgTTTT
Tnf_fw	CCCAggTTCTCTTCAAgggAC
Tnf_rv	ATACCAgggTTTgAgCTCAgC

2.1.9. Mice

In this work, female NZBxW and BALB/c mice were used. BALB/c is an albino laboratory-bred strain of House Mouse. NZBxW is a classical spontaneous model of lupus.

Female BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Female NZBxW lupus-prone mice were bred and maintained under specific pathogen-free conditions at German Rheumatism Research Centre. All mice were used for experiments at the age of either 1-2 months or 5-6 months in accordance with institutional and federal guidelines. Disease activity of NZBxW lupus-prone mice was measured using proteinuria levels. Urine samples were tested for proteinuria using Multistix 10 SG on a 0 to ++++ scale, where 0 corresponds to a negative sample, + corresponds to proteinuria of 0.3 g/L, ++ to 1 g/L, +++ to 3 g/L and ++++ to ≥ 20 g/L. Mice with proteinuria of ≥ 20 g/L were used for experiments in this work.

2.2. Cell biological methods

2.2.1. Methods of cell sorting

For the separation of T lymphocytes from PBMCs, magnetic-activated cells sorting was used. For subsequent isolation of memory T cells and their cytokine producing subsets from the whole T cell population, fluorescent-activated cells sorting was used.

Magnetic-activated cells sorting (MACS) is a technique that uses monoclonal antibodies, coupled to paramagnetic beads, which bind to surface antigens on target cells. After magnetic labeling, cell solution is transferred to a steel-wool filled column, placed in a strong magnetic field. In this step, unlabeled cells flow through, while the labeled cells stay on the column and can be easily eluted, when the column is removed from the magnetic field. This procedure allows negative and positive cell sorting, respectively.

Fluorescent-activated cells sorting (FACS) is based on the principles of flow cytometry. After the sample is hydro-dynamically focused, each cell is probed with a beam of light. The scatter and fluorescence signals are compared to the sorting criteria set on the computer. If the cell matches the selection criteria, fluid stream in form of droplets will be individually charged as it exits the nozzle of the fluidics system. Electrostatic charging occurs at a precise moment, called the “break-off point”. This is the point, when the droplet containing the cell of interest is separated from the stream and is deflected into sample tubes.

All sorting experiments in this work were performed on FACSAria and FACSAria II cell sorters at the FACS Facility of German Rheumatism Research Centre.

2.2.2. Isolation of T cells from lymphoid organs

In order to isolate T cells from lymphoid organs, mice were anesthetized with Isoflorane inhalation and euthanized by cervical dislocation. Afterwards, spleens and lymph nodes were removed and placed into ice-cold PBS. Organs were cut and pushed through a Ø212 µm cell strainer and washed by centrifugation at 300 xg 20 min at room temperature (RT). Cells were incubated with red blood cell (RBC) lysis buffer (1 ml/spleen) for 3 min at RT. Cells were washed with PBS/BSA and subsequently incubated with 20 µg/ml of anti-Fc-γR antibody (in 0.5 ml PBS/BSA/spleen) to avoid unspecific binding of antibodies. Anti-CD8, anti-Mac-1 and

anti-B220 antibodies were added to the cell solution in appropriate concentrations and incubated for further 10 min on ice. After washing with PBS/BSA, cells were incubated with goat anti-rat IgG microbeads according to the manufacturer's instructions. Cells were passed through pre-separation cell filters and depleted by using LS MACS columns (up to $2x10^9$ of total cells). The flow through was considered as T cells.

Unless otherwise mentioned, all washing steps in this work were performed by centrifugation at 300 xg 8 min at 4^oC.

2.2.3. Isolation of lymphocytes from non-lymphoid organs

Lymphocytes were isolated from non-lymphoid organs using density gradient centrifugation with Pancoll or Percoll. These are slightly hypertonic solutions, which raise the density of erythrocytes and granulocytes and cause their sedimentation to the bottom of the centrifuge vial. Lymphocytes, monocytes and platelets are not dense enough, that's why they are concentrated on the boundary layer, from where the cells can be collected by pipette aspiration.

In order to isolate lymphocytes from murine blood, the latter was collected from the heart or abdominal aorta and then diluted with heparin (5 µg/ml) and PBS. Afterwards, 10 ml of blood/PBS solution was added to 5 ml Mouse Pancoll and centrifuged at 300 xg 15 min at RT without break.

In order to isolate lymphocytes from murine kidneys, liver and lungs, these organs were perfused with ice-cold PBS via the heart. Organs were extracted and cut into small pieces and incubated with Collagenase D (5 mg/ml), Collagenase from *Clostridium histolyticum* (5 mg/ml) and DNase I (10 U/µl) in RPMI1640 medium at 37°C for 40 min under continuous rotation. This enabled enzymatic and mechanic disaggregation of the tissues, whereby the functionality and integrity of cell surface proteins remained intact. To isolate lymphocytes, organ pieces were pushed through a Ø212 µm metal cell strainer and then resuspended in 40% Percoll and overlaid onto 70% Percoll, thus creating a gradient. The samples were centrifuged at 300 xg 5 min at RT without break.

2.2.4. Isolation of memory CD4 T cells using FACS

All isolated lymphocytes from lymphoid and non-lymphoid organs were incubated with pretitrated fluorochrome-labeled antibodies against CD4, CD44, CD25 and CD62L (in 0.1 ml PBS/BSA/1x10^6 cells) for 10 min on ice. After one washing step, cells were resuspended with PBS/BSA (1 ml per max. 80x10^6 cells) and passed through pre-separation filters. Subsequently, cells were processed for sorting by flow cytometry.

$CD4^+CD25^-CD44^-CD62L^+$ cells were considered as naive T cells; $CD4^+CD25^-CD44^+CD62L^+$ cells were considered as central memory T cells (T_{CM}) and $CD4^+CD25^-CD44^+CD62L^-$ cells were considered as effector memory T cells (T_{EM}) (Figure 9).

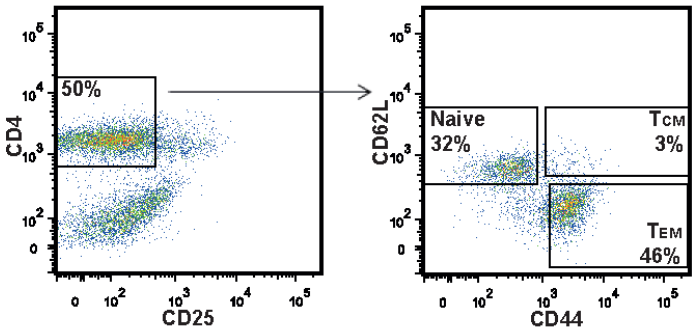

Figure 9. Sorting strategy for naive and memory CD4 T cells.
CD4 T cells were sorted into naive, central memory (T_{CM}) and effector memory T cells (T_{EM}), according to their capacity to express CD62L and/or CD44. Cells, expressing CD25, a characteristic marker for T_{REG} cells, were sorted out by negative selection.

2.2.5. Determination of cell numbers using hemocytometer

Cell numbers were counted under light microscope using a hemocytometer, also called Neubauer counting chamber, or a cell counter. Therefore, 10 µl of cell solution was pipetted into the space between the cover glass and the hemocytometer and analyzed under light microscope.

The hemocytometer consists of four corner squares. The sides of each corner square are 1 mm long, so the surface of each corner square is 1 mm^2. After covering the chamber by a cover glass, there builds up a space with 0.1 mm height, creating a volume of 0.1 mm^3, or 0.1 µl. By multiplying the amount of counted cells in each corner square with 10,000 one will get the amounts of cells in 1 ml, hence 10,000 x 0.1 µl = 1 ml.

2.2.6. Stimulation of T cells *in vitro*

In order to analyze cytokine production by memory T cells with the help of flow cytometry, $5x10^6$ memory T cells were restimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and Ionomycin (1 µg/ml) in 1 ml RPMI1640 medium and incubated for 5 h at 37°C. This kind of stimulation leads to maximal cytokine production. However, PMA and Ionomycin circumvent physiological conditions of the T cell receptor (TCR) stimulation. PMA recruits Protein kinase C (PKC), which activates via several steps IκB kinase (IKK) complex. IKK in its turn phosphorylates Inhibitor of κB (IκB), which under resting conditions sequesters Nuclear factor of κB (NF-κB). Phosphorylation of IκB leads to the ubiquitylation and degradation of IκB, allowing translocation of NF-κB into the nucleus (Lin and Weiss, 2001). In addition, Diacylglycerol (DAG) activates Ras and via several steps the Mitogen-activated protein kinase (MAPK) cascade, which stimulates Activator protein 1 (AP-1) (Lin and Weiss, 2001). Ionomycin stimulates influx of extracellular calcium into the cytoplasm. Calcium influx activates Calmodulin (CaM), which subsequently binds and thereby activates the serine/threonine phosphatase Calcineurin (Klee et al., 1998). Calcineurin in its turn dephosphorylates Nuclear factor of activated T cells (NFAT), which can then enter the nucleus (Crabtree and Olson, 2002; Scheel et al., 2012). In this way, these three transcription factors (NF-κB, AP-1 and NFAT) get activated and can migrate into the nucleus, where they bind to DNA and lead to cytokine expression (Figure 10).

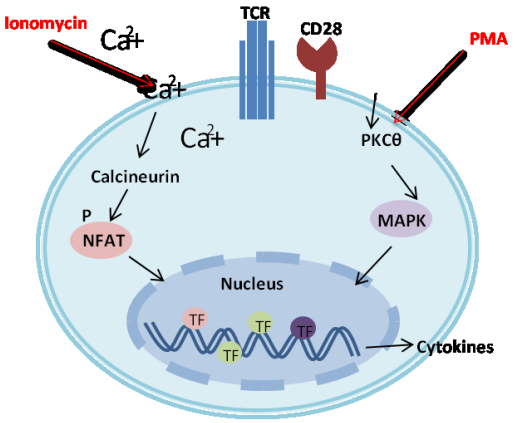

Figure 10. Stimulation of T cells via PMA and Ionomycin.
A Schematic illustration. Phorbol 12-myristate 13-acetate (PMA) activates Protein kinase C (PKC). Ionomycin induces calcium (Ca^{2+}) influx into the cell. By these stimuli, transcription factors (TF) get activated and migrate into the nucleus, where they bind to DNA and regulate expression of cytokines. TCR – T cell receptor, NFAT – Nuclear factor of activated T cells, MAPK - Mitogen-activated protein kinase.

2.2.7. Preparation of cells for the analysis by flow cytometry

Flow cytometry allows for measurement and analysis of multiple cell parameters simultaneously at a single cell level. These parameters include cell size, granularity and fluorescence intensity. The principle of flow cytometry is based on three main systems: fluidics (transport of cells to the laser beam), optics (direction of the light signal to detectors) and electronics (conversion of the light signal into electronic signals that are processed by a computer). For flow cytometric analysis, cells can be labeled with antibodies coupled to different fluorescent dyes, which can be excited with blue, red, violet and UV lasers (LSR, Fortessa).

All flow cytometric measurements in this work were performed on LSRII or Fortessa cell analyzers at the FACS Facility of German Rheumatism Research Centre. FACS data were analyzed with FlowJo 7.6.5 and R data analysis software.

2.2.7.1. Dead cell staining

In order to avoid false signals from dead cells, they were gated or sorted out prior to data analysis. For this purpose, propidium iodide (PI) was added to the cell solution according to the manufacturer's instructions. PI diffuses into the cells and is actively pumped out by living cells. In dead cells, PI intercalates into the DNA proportionally to the amount of DNA in the cell. This results in an intense fluorescent staining and allows for discrimination of viable and dead cells by flow cytometry (Figure 11). However, PI staining is not applicable to fixed cells.

For determination of the viability of cells prior to fixation, Pacific Orange LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit was used according to the manufacturer's instructions. The dye reacts with free amines only on the surface of viable cells and both on the surface and in the cytoplasm of dead cells, resulting in a more intense fluorescent staining of dead cells. This allows for discrimination of viable and dead cells by flow cytometry (Figure 11).

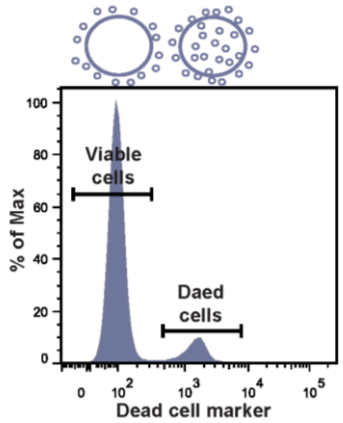

Figure 11. Discrimination of viable and dead cells by flow cytometry.
The histogram represents staining of viable and dead cells by flow cytometry. Dead cells (on the right) fluoresce more intense than viable cells (on the left), since the dye stains not only the surface molecules (like on viable cells), but also intracellular particles.

2.2.7.2. Surface staining

In order to analyze surface receptors of T cells, $1x10^6$ cells were washed with 1 ml PBS/BSA and incubated with pre-titrated fluorochrome-labeled surface antibodies (diluted in 0.1 ml PBS/BSA) for 20 min on ice. Afterwards, cells were washed with PBS/BSA and passed through pre-separation filters. Cells were processed either for the analysis by flow cytometry or for further intracellular cytokine staining.

2.2.7.3. Fixation with formaldehyde

For intracellular cytokine staining, the antibodies should be able to pass into the cell interior. Therefore, plasma membrane of cells should be permeabilized. In addition, morphological characteristics of cells should be maintained to enable their analysis by flow cytometry. For this reason, prior to permeabilization, fixation of cells is required. Formaldehyde is a cell fixative, which reacts with primary amines on proteins and nucleic acids in a cross-linking fashion to form methylene bridges, maintaining cell morphology (Fox et al., 1985).

For fixation, $1x10^6$ cells were washed with PBS and fixed with 0.5 ml of 2% formaldehyde (diluted in PBS) for 20 min at RT.

2.2.7.4. Permeabilization with saponin

After fixation, $1x10^6$cells were permeabilized with 0.5 ml of 0.5% saponin (diluted in PBS) for 5 min on ice. Saponin extracts lipids from the fixed cell membranes generating pores. These pores are large enough and allow antibody molecules to pass into the cell interior.

2.2.7.5. Intracellular cytokine staining

To enable binding of antibody molecules to cytokines in the cell, the release of cytokines from the cell should be avoided. For this purpose, Brefeldin A was used. Brefeldin A is a fungal metabolite, which interferes with vesicle transport from the endoplasmic reticulum to the Golgi apparatus, impeding cytokine release.

Isolated memory T cells were treated with Brefeldin A (1 µg/ml) for 3.5 h. Afterwards, cells were prepared for intracellular staining by fixation and permeabilization, as described above (see 2.2.7.3. and 2.2.7.4.). Pre-titrated fluorochrome-labeled antibodies were added to $1x10^6$ cells (in 0.1 ml of 0.5% saponin) and incubated for 30 min at 4^oC. Cells were washed in PBS/BSA and passed through pre-separation filters. Finally, cells were processed for the analysis by flow cytometry.

2.2.8. Cytokine secretion assay

Cytokine secretion assay allows for analysis of viable cytokine producing cells (Figure 12). In order to isolate viable IFN-γ and IL-2 producing cells, $5x10^6$ memory T cells were stimulated with PMA and Ionomycin for 5 h (see 2.2.6.). Subsequently, mouse IFN-γ and IL-2 Secretion Assay Detection Kits in APC and PE, respectively, were used according to the manufacturer's instructions. Dead cells were stained with PI (see 2.2.7.1). Cells were passed through pre-separation filters and then processed for the analysis by flow cytometry.

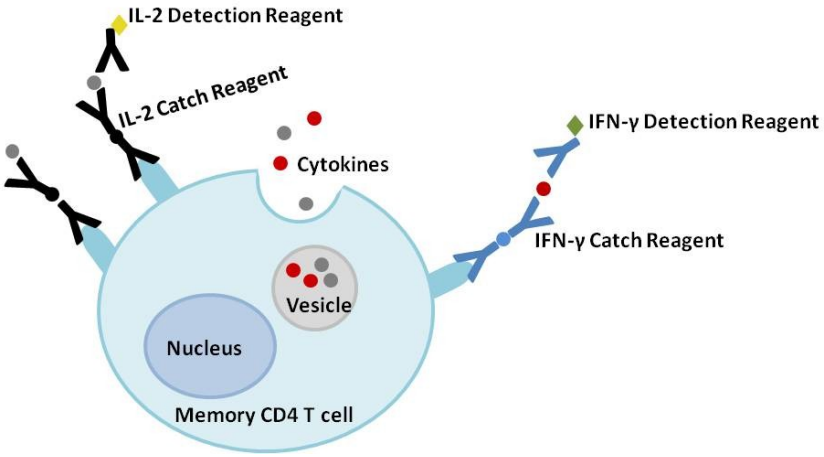

Figure 12. Cytokine secretion assay.
A schematic illustration. IL-2 and IFN-γ Catch Reagents were attached to the cell surface of stimulated memory CD4 T cells. IL-2 and IFN-γ, released by these cells could bind to their appropriate Catch Reagents and were subsequently labeled with fluorochrome-conjugated anti-IL-2 and anti-IFN-γ antibodies (or Detection Reagents), allowing for flow cytometric analysis.

2.2.9. Isolation of memory CD4 T cell subsets for gene expression analysis

In order to perform gene expression analysis, memory CD4 T cell subsets were defined, according to their capacity of IFN-γ and IL-2 production. Therefore, combined IFN-γ and IL-2 cytokine secretion assay was used (see 2.2.8.), which allowed for isolation of four cell subsets: IFN-γ^{-}IL-2^{-} double negative (DN), IFN-γ^{-}IL-2^{+} single positive (IL-2 SP), IFN-γ^{+}IL-2^{+} double positive (DP) and IFN-γ^{+}IL-2^{-} single positive (IFN-γ SP) cells (Figure 13A). Purity of isolated cell subsets was measured by flow cytometry and was more than 95%. Quality control was performed using intracellular cytokine staining for flow cytometry. The frequencies of isolated memory CD4 T cell subsets were similar in both cases: When analyzed by cytokine secretion assay and intracellular cytokine staining (Figure 13B), proofing cytokine secretion assay as a reliable technique for isolation of living cytokine producing cells.

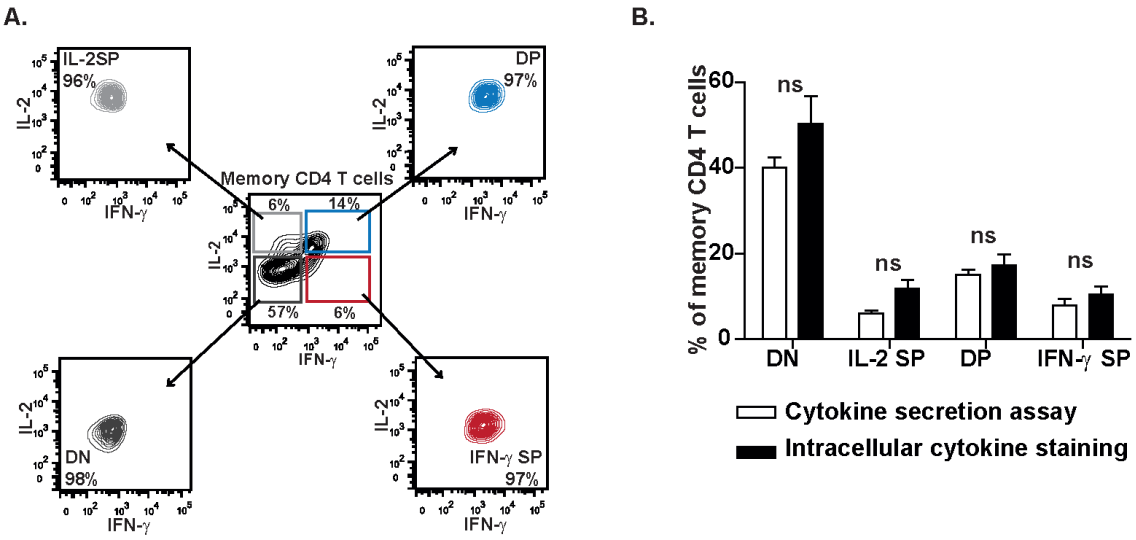

Figure 13. Isolation of memory CD4 T cell subsets.
(A) IFN-γ^-IL-2^- double negative (DN, dark grey), IFN-γ^-IL-2^+ single positive (IL-2 SP, light grey), IFN-γ^+IL-2^+ double positive (DP, blue) and IFN-γ^+IL-2^- single positive (IFN-γ SP, red) subsets were isolated from PMA and Ionomycin stimulated memory CD4 T cells ($CD4^+CD25^-CD44^+CD62L^-$) using combined IFN-γ and IL-2 secretion assay. The percentage of purity of sorted cells is depicted next to the sample names. (B) Frequencies of DN, IL-2 SP, DP and IFN-γ SP subsets were determined by cytokine secretion assay (white bars) and intracellular cytokine staining (black bars) using flow cytometry. N=5, $^{ns}p>0.05$ was determined using t-test.

2.2.10. Cultivation of memory CD4 T cell subsets *in vitro*

In order to investigate functionality of memory CD4 T cell subsets, such as their proliferative capacity, apoptosis rates as well as stability of cytokine production, PMA and Ionomycin stimulated memory CD4 T cell subsets were cultivated *in vitro* either without or with TCR stimulation by plate-bound anti-CD3 (1 µg/ml) and anti-CD28 (2 µg/ml) antibodies. Recombinant mouse IL-2 (0.01 µg/ml), IFN-γ (0.01 µg/ml) and IL-7b (0.01 µg/ml) were added to the *in vitro* cell cultures. IL-2 and IL-7 are required for proliferation and survival of memory CD4 T cells, respectively. IFN-γ was added to the *in vitro* cultures in order to maintain cytokine balance in the microenvironment, imitating the *in vivo* situation. $0.5x10^6$ cells per well were incubated in 0.5 ml RPMI1640 medium at 37°C and 5% CO_2 for up to five days. Cells were splitted on day three.

2.2.11. Proliferation assay

To determine proliferation potential of memory CD4 T cell subsets, carboxyfluorescein N-succinimidyl ester (CFSE) assay was used. CFSE crosses the plasma membrane of cells and covalently binds to intracellular molecules with its fluorochrome molecule (carboxyfluorescein). When a CFSE-labeled cell divides, its progeny will get the half of the amount of the dye, which will result in sequential halving of the initial fluorescence. This assay allows for visualization and analysis of cell division by flow cytometry (Figure 14).

$1x10^6$ cells were stained with 5 µM CFSE for 2.40 min at RT and subsequently washed with PBS. Then, cells were cultivated as described in 2.2.10. Division index and percent of dividing cells were calculated by proliferation tool provided by FlowJo data analysis software.

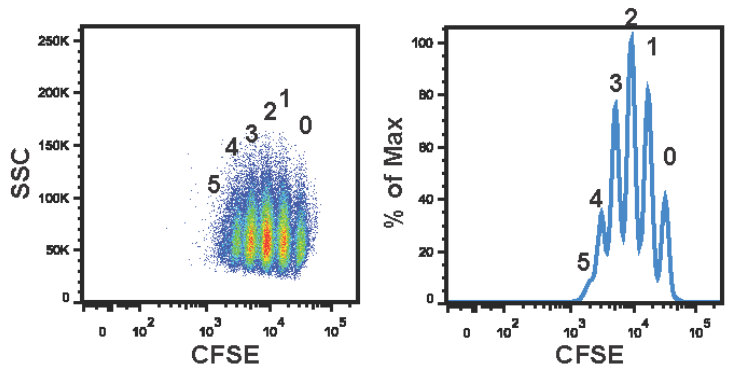

Figure 14. Visualization of cell proliferation.
Pseudocolor diagram (on the left) and histogram (on the right) show division of cells labeled with carboxyfluorescein N-succinimidyl ester (CFSE). Each division results in sequential halving of the initial fluorescence of CFSE. Initial fluorescence peak is indicated as 0. Peaks, indicated as 1, 2, 3, 4 and 5 represent following generations of cells.

2.2.12. Ki-67 staining

In order to determine proliferation of memory CD4 T cell subsets, intracellular Ki-67 staining for flow cytometry was used according to the manufacturer's instructions. Ki-67 is an antigen, which is present in active phases of cell cycle (G_1, S, G_2 and M) and is absent in resting cells (G_0). Ki-67 is commonly used as a proliferation marker, especially in oncology (Gerdes et al., 1983). Positive Ki-67 staining indicates mitotically active cells, whereas negative Ki-67 staining indicates resting cells (Figure 15).

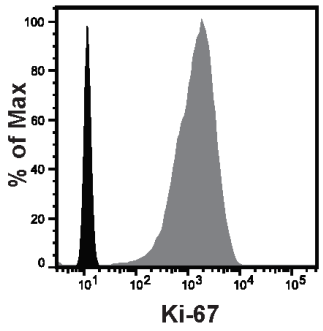

Figure 15. Ki-67 staining for flow cytometry.
The histogram represents intracellular staining of memory CD4 T cells with anti-Ki-67 antibody for flow cytometry. Resting cells show negative Ki-67 staining (black histogram). Cells, *in vitro* cultivated for 5 days in the presence of TCR stimulation reveal positive Ki-67 staining (grey histogram), indicating their proliferative activity.

2.2.13. Apoptosis assay

In order to determine apoptosis potential of memory CD4 T cell subsets, *in vitro* cultivated cells were stained with Annexin V Apoptosis Detection Set PE-Cyanine7 and propidium iodide (PI) according to the manufacturer's instructions. In early-stage apoptosis phosphatidylserine, which is normally located in the inner leaflet of the plasma membrane, is translocated to the extracellular membrane leaflet marking cells as targets of phagocytosis. In this stage, Annexin V binds phosphatidylserine in a calcium-dependent manner, while PI is excluded by the plasma membrane. These early-stage apoptotic cells will stain Annexin V, but not PI. In late-stage apoptosis, or necrosis, the cell membrane loses its integrity allowing both Annexin V and PI to access into the interior of the cell. These necrotic cells will stain both Annexin V and PI. In this way, apoptotic and dead (necrotic) cells can be distinguished from each other by flow cytometry (Figure 16).

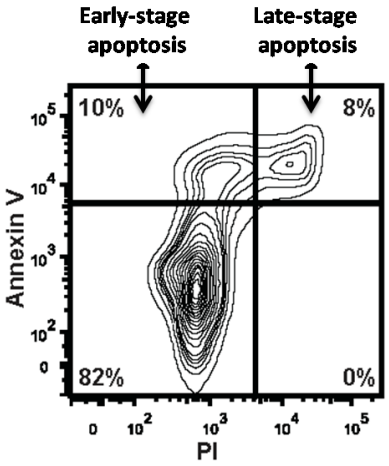

Figure 16. Discrimination of apoptotic cells from dead cells.
The contour diagram shows distribution of apoptotic and dead cells, according to Annexin V and propidium iodide (PI) staining. Early-stage apoptotic cells stain Annexin V, but not PI, whereas late-stage apoptotic, or dead cells, stain both Annexin V and PI.

2.3. Molecular biological methods

2.3.1. Microarrays

Microarray technology enables global analysis of gene expression during cellular processes. In one experiment, thousands of genes can be investigated simultaneously. Microarray is a glass slide, on which DNA molecules are fixed in an orderly manner (or spots).

Microarray procedure works as follows: First, RNA is extracted from the cells and then reverse transcribed into cDNA by reverse transcriptase. Subsequently, cDNA from different samples is labeled with different fluorescent dyes. After labeling, cDNA is hybridized on the same glass slide to its complementary sequence. Finally, the spots are excited by a laser and scanned to detect the fluorescent dyes. The amount of fluorescence emitted is proportional to the amount of bound nucleic acid. Thus, each spot of the microarray corresponds to a gene and its fluorescence value represents relative expression level of that gene.

In this work, distinct subsets of memory CD4 T cells of diseased NZBxW lupus-prone mice were analyzed with Affymetrix GeneChip MoGene 2.0 ST microarrays for three independent replicates according to the manufacturer's instructions.

2.3.2. RNA purification

For microarray analysis, first total RNA was isolated from memory CD4 T cell subsets using RNeasy Plus Mini Kit according to the manufacturer's instructions. With this kit, samples were first lysed and homogenized and RNases were inactivated, then genomic DNA was removed. Administration of ethanol enabled appropriate binding of RNA to the membrane of the spin column. Subsequently, RNA was eluted in 33 µl of water. RNA concentration was measured using NanoDrop ND-1000 Spectrophotometer and quality control was performed at Bioanalyzer. Samples with RNA integrity numbers (RIN) of ≥ 8.9 were used for subsequent gene expression analysis. Finally, total RNA was processed, amplified, labeled and hybridized to Affymetrix GeneChip MoGene 2.0 ST microarrays according to the manufacturer's instructions.

RNA purification was performed for three independent replicates. Numbers of DN, IL-2 SP, DP and IFN-γ SP cells and amounts of RNA, isolated from these cell subsets are shown in Table 11. 100 ng of RNA from each sample was used for microarray analysis.

Table 11. Numbers of DN, IL-2 SP, DP and IFN-γ SP cells and corresponding amounts of isolated RNA

Sample name	Cell number	RNA amount ng/µl
DN_1	5,500,000	32
DN_2	4,700,000	32
DN_3	2,200,000	39
IL-2 SP_1	920,000	18
IL-2 SP_2	1,300,000	24
IL-2 SP_3	540,000	3.7
DP_1	1,900,000	41
DP_2	3,800,000	57
DP_3	1,400,000	46
IFN-γ SP_1	1,200,000	18
IFN-γ SP_2	800,000	5.5
IFN-γ SP_3	740,000	3.5

2.3.3. cDNA synthesis

In order to validate the results of microarray data by quantitative real-time polymerase chain reaction (qRT-PCR), PCR products were generated from total RNA. Therefore, total RNA was reverse transcribed into cDNA using Reverse Transcriptase Superscript II-Kit according to the manufacturer's instructions. Reverse transcriptase is RNA-directed DNA polymerase. This enzyme synthesizes a complementary DNA (cDNA) strand from single-stranded RNA. cDNA was next used as a template for amplification by PCR.

2.3.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

PCR is a technique for amplifying DNA. An enzyme (polymerase) synthesizes multiple sequences of DNA complementary to the gene of interest. To enable this, short DNA sequences (primers) bind to the start and the end of the DNA target, priming DNA sample for polymerase activity.

For optimal working of PCR, a specific temperature curve has to be applied: 95°C (denaturation: separation of double stranded DNA into single strands); 60°C (annealing: binding of primers to DNA); 72°C (elongation: copying DNA strands by polymerase). These temperature changes are repeated for 40 cycles, creating billions of copies. In addition, DNA-binding fluorescent dye is included in the reaction. Its signal increases proportionally to the amount of DNA with each cycle.

qRT-PCR was performed for duplicates in three independent experiments in 96-well-formate using Stratagene Mx3005P and Qiagen SYBRGreen Kit according to the manufacturer's instructions. Gene-specific oligodeoxynucleotides (primers) were synthesized by TIB Molbiol (Berlin, Germany) and are presented in Table 11. mRNA expression levels of target genes were normalized to mRNA expression levels of *ribosomal protein S18 (rps18)* reference gene. For quantification of gene expression, ΔCT method was used (Livak and Schmittgen, 2001).

2.3.5. Quality analysis of current Affymetrix Gene Chips dataset

The analysis of microarray data, such as quality controls, data normalization, sample correlation, statistical analyses of differences in gene expression as well as the assessment of the randomness of the observed overlaps between two gene lists were performed by Dr. Paul Hammer (Ibiomics GmbH, Berlin, Germany).

To determine, if any anomalies existed in current microarray data, an optical control of all image plots was performed. Thereby, no spatial artifacts or other non-homogeneous patterns were seen in the image plots.

All image plots appeared similar to each other and displayed no obvious anomalies, except for the black point and the white area in the centre of the images (Figure 17). These two features appeared in all images and seemed to be a systematically artifact without any influence on the measurement.

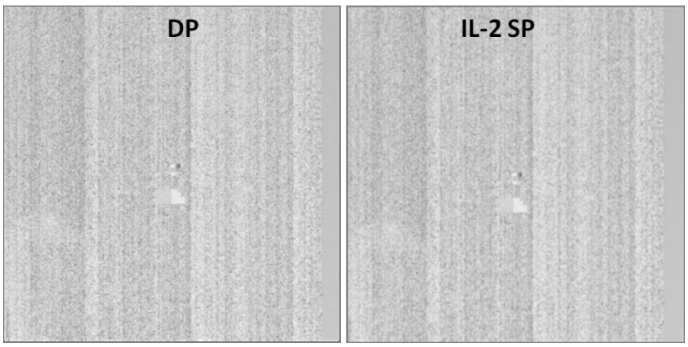

Figure 17. Raw image plots of microarray data.
Two example images of raw microarray data are presented. All image plots are characterized by a homogenous grey pattern with a black point and a white area in the centre of the images. On the top of images the sample names are given. Data were analyzed by R software. N=3.

To further determine existence of potentially defective arrays, density plots and boxplots of probe-level data were analyzed. In conformity to the image plots, all density plots and boxplots of raw data appeared similar to each other and displayed no obvious anomalies (Figure 19A).

The last quality assessment was performed with the MA plot. When two microarrays are being compared, difference of log intensities for each probe on each gene is plotted against their average. The log ratios of two measurements are called M values (from “minus” in the log scale) and are represented in the vertical axis. The mean values of two measurements are called A values (from “average” in the log scale) and are represented in the horizontal axis. Figure 18 shows MA plots for array comparisons of two samples (DN_1 vs. DN_2 and DP_1 vs. DN_1), whereby the red lines show a loess regression line. Quality problems are most apparent from the MA plot. For example, if the loess smoother oscillated wildly or if the variability of the M values appeared to be greater in one or more arrays relative to the others. No such anomalies occurred in any biological replicated data.

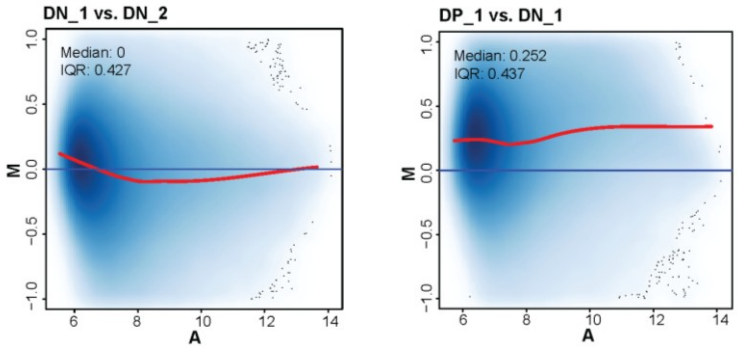

Figure 18. MA plots for the array comparisons.
MA plots show comparison of two samples (DN_1 vs. DN_2 and DP_1 vs. DN_1). The difference of log intensities for each probe on each gene ('M') is plotted against their average ('A').

Thus, in all quality control images and plots no serious anomalies were seen, indicating that this Affymetrix Gene Chips dataset had no quality issues.

2.4. Statistical methods

2.4.1. Normalization of microarray data by RMA method

For analysis of microarray data, R software was used. In particular, "xps" package was used for reading, visualization, annotation and transformation of raw Affymetrix GeneChip MoGene 2.0 ST microarray data (Stratowa, 2014).

Raw probe-level data were transformed into final expression values for each gene (or Affy IDs) by multichip averaging method (RMA) (Figure 19B). RMA performs a convolution background correction, quantile normalization and summarization, based on a multi-array model fit robustly using the median polish algorithm (Bolstad et al., 2005). This algorithm uses only perfect match (PM) information (Irizarry et al., 2003).

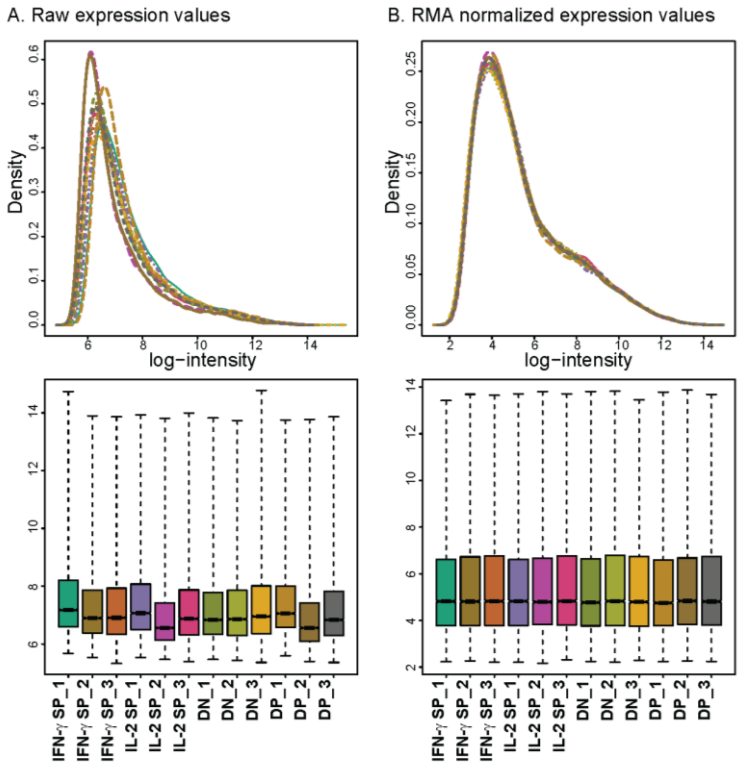

Figure 19. Probe-level data of microarray analysis.
Density plots (upper panel) and boxplots (lower panel) of raw probe-level data (A) were normalized by RMA method (B). Numbers (1, 2, 3) next to the sample names represent three independent replicates.

2.4.2. Correlation analysis of gene expression data

Analysis of gene expression data is principally based on comparisons of gene expression profiles between given samples. For this comparison, Principal Coordinates Analysis (PCoA) and Pearson Correlation Coefficient (PCC) were used in this work to quantify similarities/dissimilarities and correlations of gene expression profiles of the samples, respectively. For classical multidimensional scaling of the distance data matrix, cmdscale() function was used. Correlation distances were calculated with dist() function. Both functions were given by "stats" package of R software (Gower, 1966). For calculation and visualization of correlation values, cor() function provided by "stats" package of R-script 'myImagePlot.R' from www.phaget4.org were used.

2.4.3. Statistical analysis of differences in gene expression

In order to identify differences in gene expression between samples, gene expression values were transformed into the binary logarithm (log2) and moderated t-statistics were used to

determine the significance of differences in gene expression. Therefore, “limma” package in Bioconductor (Ritchie et al., 2015) was used, which provides the fold change value, the average expression value and the p-value for every gene and comparison (Benjamini and Hochberg, 1995). It is based on an empirical Bayes approach, described in detail by Huber et al. and Smyth (Huber et al., 2005; Smyth, 2005). All genes with a p-value under 0.05 were considered as significantly differentially expressed genes.

2.4.4. Student's t-test

Significance between differentially expressed genes was determined with the help of type 3 student’s t-test. Thereby, mistake probability of $p<0.05$ was considered as significant. Two sample groups were assumed to be normally distributed with unequal variance. Calculation was performed using the t.test() function from the “stats” package of R software. Significance of protein expression data was calculated using t-test function of the GraphPrism software.

2.4.5. Fisher's exact test

In order to calculate significance values of the overlap of two gene lists in comparison to the whole genome, Fisher’s exact test was used. Mistake probability of $p<0.05$ was considered as significant. Calculation was performed using fisher.test() function of “stats” package of R software.

2.4.6. Venn diagrams

Venn diagrams were created in order to visualize the overlaps of two or more independent gene lists. Therefore, online tool “Venn Diagrams”, provided by the working group of Bioinformatics & Evolutionary Genomics of the University of Gent was used from http://bioinformatics.psb.ugent.be/webtools/Venn.

2.4.7. Simulation of random overlap of two gene lists

For the assessment of the randomness of the observed overlap of two or more gene lists, probability diagrams were created by the 100,000-fold random repetition. Afterwards,

randomly created overlap of dragged genes from gene lists A and B was determined and the probability of the size of the overlap was calculated and visualized. Calculation was performed by a self-created program in R software (author Dr. Paul Hammer).

2.4.8. Gene enrichment analysis of microarray data

Gene Ontology (GO) analysis of microarray data was performed by the database for annotation, visualization and integrated discovery (DAVID6.7) using classic algorithm and modified Fisher's test (Huang et al., 2009a; Huang et al., 2009b). *Mus musculus* was used as background for the analysis. Significantly ($p<0.05$) up- and downregulated genes in the comparison between IFN-γ SP and DP cells were used for the analysis. With top 10 of enriched BP (biological process) GO terms was operated.

2.4.9. Three-parameter visualization of flow cytometry data

Three-parameter visualization tool for the analysis of flow cytometry data was recently created using R software in the “Signal Transduction” working group at the German Rheumatism Research Centre. For Three-parameter visualization, flow cytometry data were manually compensated and gated in FlowJo 7.6.5 data analysis software. Then, data were exported into regular csv tables, where each column contained fluorescence intensity values of one cell parameter, labeled with a specific fluorescent dye, and each row contained individual cells. These tables were used as an input for a customized R script. Columns with staining information were transformed with inverse hyperbolic sine, which is a common transformation method for flow cytometry data (Finak et al., 2010). Three-parameter visualization tool was established to quantify frequencies of three given cell parameters (or proteins) simultaneously. Fluorescent intensities of cell parameters 1 and 2 were divided into two-dimensional bins and plotted on the x and y axis, respectively. In this case, it was adjusted that each bin contained at least 20 cells. Based on staining controls, a threshold of the fluorescent intensity value for the 3rd parameter was determined. Based on this threshold, frequencies of parameter 3 were calculated for every bin and plotted as a color-coded heat map (from 0% (blue) to 100% (red)) (Figure 20).

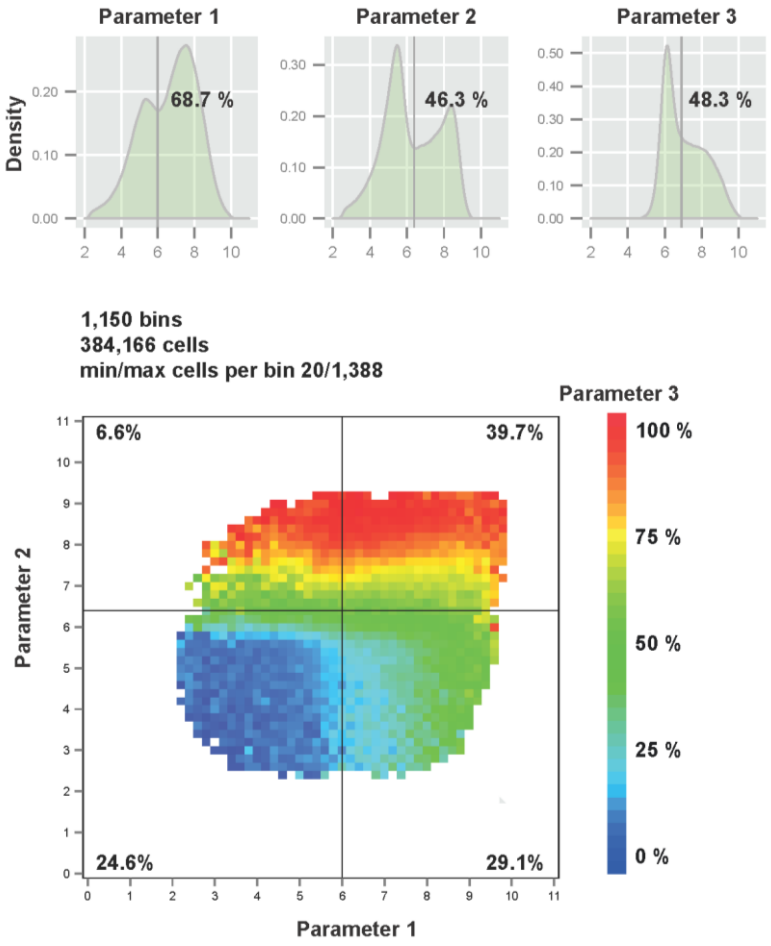

Figure 20. Three-parameter visualization of flow cytometry data.
Three cell parameters, or proteins, indicated as parameter 1, 2, 3 were labeled with specific fluorescent dyes. Fluorescent intensities of proteins 1 and 2 were divided into two-dimensional bins and plotted on x and y axis, respectively. Frequency of protein 3 was calculated and plotted in the third dimension into a color-coded heat map. Data were asin h transformed with R software. This diagram contains 1,150 bins and 384,166 cells. Each bin was set up to contain min 20 and max 1,388 cells.

3. Results

3.1. Balance of naive and memory CD4 T cells of diseased NZBxW lupus-prone mice

3.1.1. Increased frequencies of memory CD4 T cells in lymphoid organs

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, which is characterized by loss of self-tolerance and abnormal T and B cell responses (Bertsias et al., 2010). Uncontrolled activation of T and B cells is mainly mediated by cytokines (Enghard et al., 2006). In fact, numerous studies showed that production of many cytokines is altered in SLE (Smolen et al., 2005). In this work, alterations of two cytokines produced by CD4 T cells, interleukin-2 (IL-2) and interferon-gamma (IFN-γ), were closely investigated. Whereas IFN-γ is primarily produced by memory CD4 T cells (Aune et al., 1997; Sanders et al., 1988), IL-2 can be produced by both naive and memory CD4 T cells (Leonard, 2001; Setoguchi et al., 2005). Diminished production of IL-2 in SLE has been shown to contribute to the disease pathogenesis through the impairment of T_{REG} homeostasis (Humrich et al., 2015; von Spee-Mayer et al., 2016). In contrast to IL-2, the role of IFN-γ in the pathogenesis of SLE is controversial, since some studies reported about increased IFN-γ serum levels of SLE patients and its correlation with disease activity (al-Janadi et al., 1993; Masutani et al., 2001; Tokano et al., 1999; Uhm et al., 2003), and other studies reported about decreased IFN-γ serum levels in patients with active SLE (Horwitz et al., 1998; Min, 2001). Also, the data from SLE mice were not uniform, demonstrating both protective and damaging roles of IFN-γ in SLE (Nicoletti et al., 2000; Schmidt et al., 2015).

In most studies, cytokine alterations in SLE were analyzed either by measuring cytokine serum levels or analyzing average cytokine expression by peripheral blood mononuclear cells (PBMCs), which include lymphocytes, monocytes, natural killer cells (NK cells) and dendritic cells (Kleiveland, 2015). However, measuring average cytokine levels does not reflect the true impact of distinct cell types, involved in altered cytokine production. The hypothesis of this work was that memory CD4 T cells may significantly contribute to the altered production of IL-2 and IFN-γ in SLE. Therefore, one main aim of this work was to identify phenotype and functional changes of IL-2 and IFN-γ producing memory CD4 T cells of diseased NZBxW lupus-prone mice. As known, NZBxW mouse model most closely resembles human SLE (Perry et al., 2011).

First, to characterize CD4 T cell pool of diseased NZBxW lupus-prone mice in lymphoid tissues, the balance between naive and memory CD4 T cells, isolated from spleens and lymph nodes of these animals was determined. Disease activity of NZBxW lupus-prone mice was defined by age (5-6 months) and high levels of proteinuria (over 20 g/L). As a control group, age-matched healthy BALB/c mice were used.

Diseased NZBxW lupus-prone mice exhibited overall lower frequencies of CD4 T cells than healthy BALB/c mice (54% vs. 69%, respectively) (Figure 21). In addition, diseased NZBxW lupus-prone mice showed an altered composition of CD4 T cell pool as they displayed significantly increased frequencies of effector memory T cells (T_{EM}) ($CD4^+CD44^+CD62L^-$; $p<0.0001$) and significantly decreased frequencies of naive T cells ($CD4^+CD44^-CD62L^+$; ($p<0.0001$). The frequencies of central memory T cells (T_{CM}) ($CD4^+CD44^+CD62L^+$), however, were not changed between healthy BALB/c controls and diseased NZBxW lupus-prone mice (Figure 21).

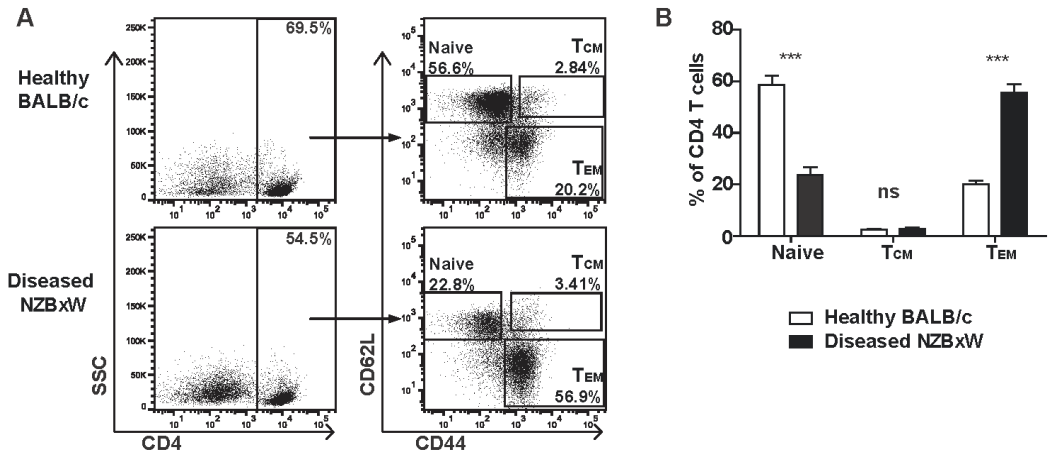

Figure 21. Increased frequencies of memory CD4 T cells in lymphoid organs of diseased NZBxW lupus-prone mice.
(A) Dot-plot diagrams show the gating strategy for naive ($CD4^+CD44^-CD62L^+$), central memory (T_{CM}) ($CD4^+CD44^+CD62L^+$) and effector memory T cells (T_{EM}) ($CD4^+CD44^+CD62L^-$) from whole CD4 T cells, isolated from spleens and lymph nodes of healthy BALB/c (upper panel) and diseased NZBxW lupus-prone mice (lower panel). (B) Bar diagram shows the percentages of naive, T_{CM} and T_{EM} cells from whole CD4 T cells of healthy (white bars) and diseased mice (black bars). N=5 with a pool of up to 10 mice per group and experiment. Significance was determined by t-test (***$p<0.0001$, $^{ns}p>0.05$).

SLE is a systemic autoimmune disease with an involvement of multiple organs, whereby lupus nephritis represents most common and probably most sever complication of this disease (Lee

et al., 2011). On this basis, the balance of naive and memory CD4 T cells was determined not only in lymphoid tissues, but also in visceral organs and blood of these animals.

3.1.2. Increased frequencies of memory CD4 T cells in non-lymphoid organs

In order to determine the balance of naive and memory CD4 T cells in non-lymphoid target organs, T cells were analyzed in blood, liver, lungs and kidneys of NZBxW lupus-prone mice before and after onset of lupus nephritis (LN). Disease onset was defined by age and proteinuria parameters. Mice at the age of 1-2 months before onset of LN displayed no proteinuria (0 g/L), whereas mice at the age of 5-6 months after onset of LN had massive proteinuria of more than 20 g/L.

Before onset of LN, naive CD4 T cells tended to prevalent in these organs, especially with regard to lungs and blood (Figure 22C), whereas after onset of LN, the frequencies of memory CD4 T cells increased in all target organs (Figure 22B).

However, due to the requirement of large cell numbers for this particular experiment, two experiments were performed, which did not allow for determination of the significance.

In summary, these experiments showed an altered balance of CD4 T cell pool in diseased NZBxW lupus-prone mice with increased frequencies of memory CD4 T cells both in lymphoid (Figure 21) and non-lymphoid organs (Figure 22).

The next aim of this work was to investigate, whether the increased numbers of memory CD4 T cells were associated with an imbalanced IFN-γ and IL-2 production in SLE. To that aim, the pattern of IFN-γ and IL-2 production among memory CD4 T cells was determined.

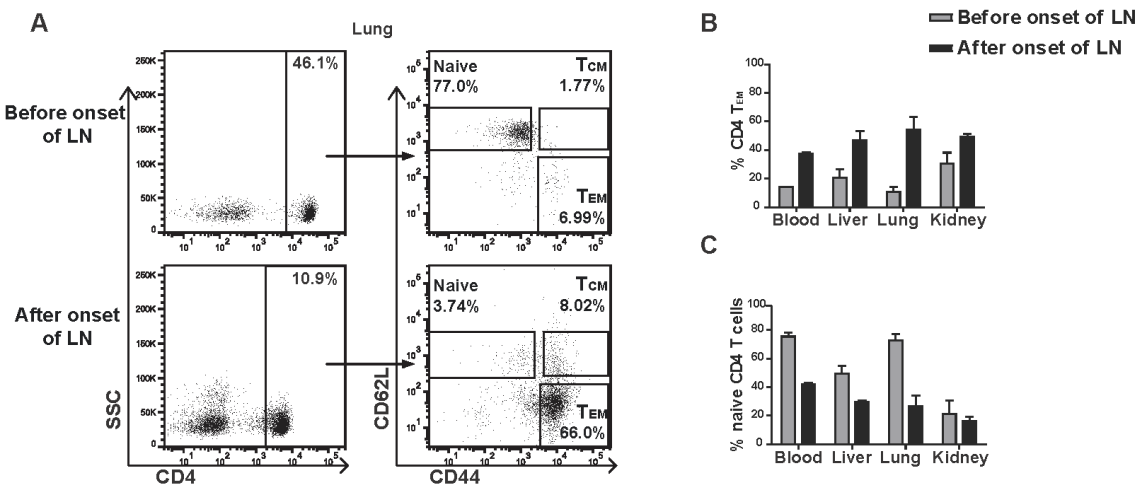

Figure 22. Increased frequencies of memory CD4 T cells in non-lymphoid organs of diseased NZBxW lupus-prone mice.
(A) Dot-plot diagrams show the gating strategy for naive ($CD4^+CD44^-CD62L^+$), central memory (T_{CM}) ($CD4^+CD44^+CD62L^+$) and effector memory T cells (T_{EM}) ($CD4^+CD44^+CD62L^-$) from whole CD4 T cells, isolated from lungs of NZBxW lupus-prone mice before (upper panel) and after onset of lupus nephritis (LN) (lower panel). (B) and (C) bar diagrams show the percentages of T_{EM} and naive T cells, respectively, from blood, liver, lungs and kidneys of NZBxW lupus-prone mice before (grey bars) and after onset of LN (black bars). N=2 with a pool of 10 mice per group and experiment.

3.2. Altered pattern of IFN-γ and IL-2 production in diseased NZBxW lupus-prone mice

3.2.1. Increased frequencies of IFN-γ SP cells in lymphoid organs

Numerous studies demonstrated alterations in cytokine production and found a correlation with SLE activity (Smolen et al., 2005). Of a particular interest for this work was the altered production of IFN-γ and IL-2 by memory CD4 T cells in SLE.

Already early studies showed diminished IL-2 production by T cells in mice (Altman et al., 1981) and humans with SLE (Alcocer-Varela and Alarcón-Segovia, 1982; Linker-Israeli et al., 1983) and its protective role in the pathogenesis of SLE (von Spee-Mayer et al., 2016). In contrast, the role of IFN-γ in the pathogenesis of SLE is not clear yet. Thus, increased IFN-γ production by T cells was shown to correlate with disease activity in NZBxW lupus-prone mice (Jacob et al., 1987), whereas in patients with active SLE both high (Uhm et al., 2003) and low (Min et al., 2001) serum levels of IFN-γ were reported.

In order to investigate the pattern of IFN-γ and IL-2 production by memory CD4 T cells, flow cytometric analysis of intracellular cytokine staining was used. Memory CD4 T cells were divided into four subpopulations, dependent on their capacity of IFN-γ and IL-2 production. These subpopulations are: IFN-γ^{-}IL-2^{-} double negative (DN), IFN-γ^{-}IL-2^{+} single positive (IL-2 SP), IFN-γ^{+}IL-2^{+} double positive (DP) and IFN-γ^{+}IL-2^{-} single positive (IFN-γ SP) cells (Figure 23).

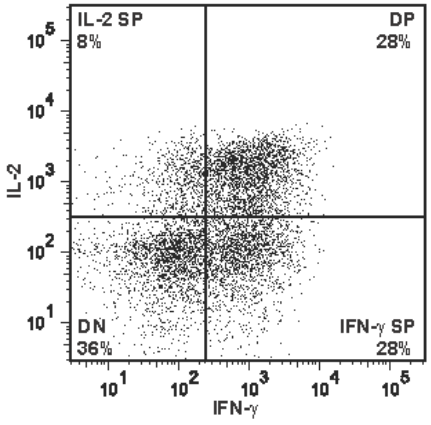

Figure 23. Distribution of DN, IL-2 SP, DP and IFN-γ SP cells.
Dot-plot diagram represents distribution of IFN-γ^{-}IL-2^{-} double negative (DN), IFN-γ^{-}IL-2^{+} single positive (IL-2 SP), IFN-γ^{+}IL-2^{+} double positive (DP) and IFN-γ^{+}IL-2^{-} single positive (IFN-γ SP) cell subsets of memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice and stimulated with PMA and Ionomycin for 5 h. Cells were analyzed by flow cytometry using intracellular cytokine staining.

To date, cytokine production in SLE was investigated in whole CD4 T cells, but not in isolated memory CD4 T cells. It is known that IFN-γ is predominantly produced by memory CD4 T cells (Sanders et al., 1988), whereas IL-2 can be produced by both naive and memory CD4 T cells (Leonard, 2001; Setoguchi et al., 2005). Since the data of current work showed increased frequencies of memory CD4 T cells in diseased NZBxW lupus-prone mice (Figure 21), the next aim of this work was to define the magnitude of IFN-γ and IL-2 production among whole CD4 T cells (including naive, memory and T_{REG} cells) and isolated memory CD4 T cells.

Flow cytometric analysis of intracellular cytokine staining confirmed that memory CD4 T cells were the main source of IFN-γ production, as they showed a 2-fold increase in IFN-γ producers,

when compared to whole CD4 T cells in both healthy and diseased mice. As expected, the overall production of IL-2 was not significantly changed comparing whole CD4 T cells and memory CD4 T cells in healthy mice. Importantly, this was true also for diseased NZBxW lupus-prone mice (Figure 24).

In order to characterize the pattern of IFN-γ and IL-2 production by memory CD4 T cells in more detail, the frequencies of DN, IL-2 SP, DP and IFN-γ SP cells were compared in whole CD4 T cells and memory CD4 T cells of diseased NZBxW lupus-prone mice and healthy BALB/c controls.

The results showed that the frequencies of IFN-γ SP cells were significantly higher in diseased NZBxW lupus-prone mice, when compared to healthy BALB/c mice: 11% vs. 4% among whole CD4 T cells ($p<0.01$), and 24% vs. 7% among memory CD4 T cells ($p<0.001$), respectively. In contrast, the frequencies of IL-2 SP cells were significantly lower in diseased NZBxW lupus-prone mice than in healthy BALB/c mice: 20% vs. 35% among whole CD4 T cells ($p<0.001$), and 10% vs. 38% among memory CD4 T cells ($p<0.001$), respectively. Whereas the frequencies of DP cells among whole CD4 T cells were slightly decreased in diseased NZBxW lupus-prone mice compared to healthy BALB/c mice: 15% vs. 18% ($p<0.05$), respectively, the frequencies of DP cells among memory CD4 T cells were not significantly changed between diseased and healthy mice: 24% vs. 29% ($p>0.05$), respectively. Whereas the percentage of DN cells among whole CD4 T cells was significantly higher in diseased NZBxW mice than in healthy BALB/c mice: 60% vs. 42% ($p<0.001$), respectively, the percentage of DN cells among memory CD4 T cells was not significantly different between diseased and healthy mice: 42% vs. 34% ($p>0.05$), respectively (Figure 24).

Taken together, the results showed overall increased production of IFN-γ and decreased production of IL-2 in diseased NZBxW lupus-prone mice both among whole CD4 T cells and memory CD4 T cells. Besides, these data confirmed prominent IFN-γ production by memory CD4 T cells and approximately equal IL-2 production by both whole CD4 T cells and memory CD4 T cells in healthy BALB/c mice and showed a similar distribution in diseased NZBxW lupus-prone mice. Interestingly, diseased NZBxW lupus-prone mice exhibited an altered pattern within memory CD4 T cell subpopulations, i.e. significantly increased frequencies of IFN-γ SP cells and diminished frequencies of IL-2 SP cells compared to healthy controls. In contrast, frequencies of DP as well as DN cells were stable between diseased NZBxW lupus-prone and healthy BALB/c mice.

Hence, these observations indicate that IFN-γ SP cell subset of memory CD4 T cells might significantly contribute to increased IFN-γ production in diseased NZBxW lupus-prone mice.

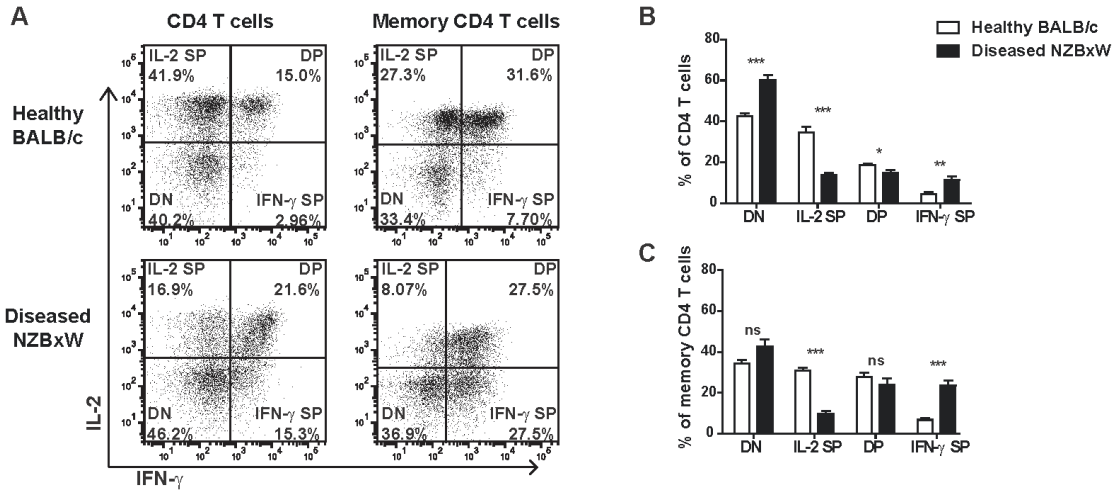

Figure 24. Increased frequencies of the IFN-γ SP cell subset of memory CD4 T cells of diseased NZBxW lupus-prone mice.

(A) Dot-plot diagrams show the gating strategy for DN, IL-2 SP, DP and IFN-γ SP cells of healthy BALB/c (upper panel) and diseased NZBxW lupus-prone mice (lower panel) in whole CD4 T cells (left panel) and memory CD4 T cells (right panel). Bar diagrams show frequencies of DN, IL-2 SP, DP and IFN-γ SP cells in (B) whole CD4 T cells and (C) memory CD4 T cells, isolated from spleens and lymph nodes of healthy BALB/c (white bars) and diseased NZBxW lupus-prone mice (black bars). Cells were stimulated with PMA and Ionomycin for 5 h and analyzed by flow cytometry using intracellular cytokine staining. N=4-6 with a pool of up to 10 mice per group and experiment. Significance was determined by t-test (***$p<0.001$, **$p=0.01$, *$p<0.05$, $^{ns}p>0.05$).

3.2.2. Increased amounts of IFN-γ SP cells in non-lymphoid organs

As shown in this work, the frequencies of memory CD4 T cells were increased not only in lymphoid (Figure 21), but also in non-lymphoid target organs (Figure 22), therefore, the next aim of this work was to investigate the balance of IL-2 and IFN-γ production by memory CD4 T cells in non-lymphoid target organs, as well.

In order to analyze, whether increased frequencies of memory CD4 T cells were associated with altered cytokine production in non-lymphoid organs, the amounts of DN, IL-2 SP, DP and IFN-γ SP cells were measured in blood, liver, lungs and kidneys of NZBxW lupus-prone mice before and after onset of lupus nephritis (LN) using flow cytometric analysis of intracellular cytokine staining. The amounts of DN, IL-2 SP, DP and IFN-γ SP cells were calculated from the

whole amounts of memory CD4 T cells, isolated from each organ. Thereby, the amounts of memory CD4 T cells were equal between both mice groups (before and after onset of LN). High levels of proteinuria (over 20 g/L) in mice at the age of 5-6 months were used as an indicator of sever nephritis. Mice at the age of 1-2 months before onset of LN displayed no proteinuria (0 g/L).

Before onset of LN, very low (almost irrelevant) amounts of IFN-γ and IL-2 producers were identified among memory CD4 T cells in non-lymphoid organs (blood, liver, lungs and kidneys). This changed drastically in diseased NZBxW lupus-prone mice, as the production of both IFN-γ and IL-2 was readily detectable within the memory CD4 T cell population of target organs (Figure 25).

However, due to the requirement of large cell numbers for this particular experiment, two experiments were performed, which did not allow for determination of the significance.

Nevertheless, these observations suggest that increased frequencies of memory CD4 T cells, which infiltrated target organs particularly in diseased NZBxW lupus-prone mice were associated by increased overall cytokine production, which in its turn might point to their activated phenotype.

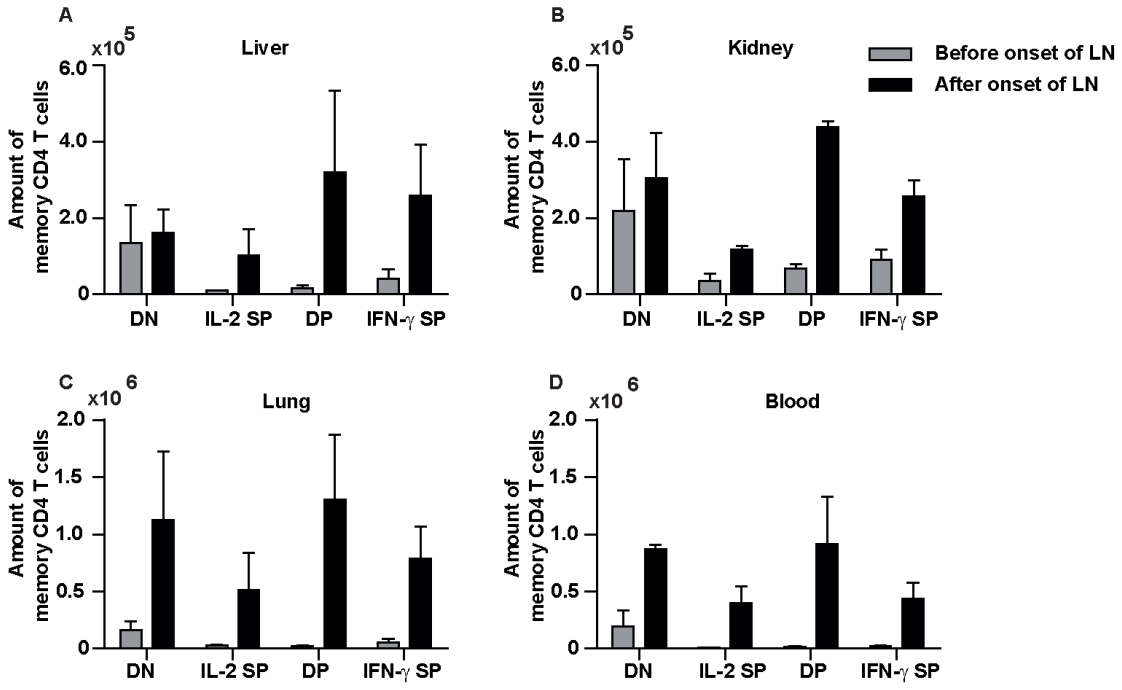

Figure 25. Increased IFN-γ and IL-2 production by memory CD4 T cells in target organs of diseased NZBxW lupus-prone mice.

Bar diagrams represent the amounts of DN, IL-2 SP, DP and IFN-γ SP cells in (A) liver, (B) kidneys, (C) lungs and (D) blood of NZBxW lupus-prone mice before (grey bars) and after (black bars) onset of lupus nephritis (LN). Amounts of DN, IL-2 SP, DP and IFN-γ SP cells were determined from the whole amounts of memory CD4 T cells, isolated from each organ. Memory CD4 T cells were stimulated with PMA and Ionomycin for 5 h and analyzed by flow cytometry using intracellular cytokine staining. N=2 with a pool of 10 mice per group and experiment.

3.3. Gene expression of memory CD4 T cell subsets of diseased NZBxW lupus-prone mice

3.3.1. Specific gene expression profiles of DN, IL-2 SP, DP and IFN-γ SP cells

As shown in this work, the frequencies of IFN-γ SP cell subset of memory CD4 T cells were significantly increased in lymphoid organs of diseased NZBxW lupus-prone mice (Figure 24C). In this context, the next aim of this work was to characterize this subset of memory CD4 T cells. For this specific issue, memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were divided into four subpopulations, according to their capacity of IFN-γ and IL-2 production into DN, IL-2 SP, DP and IFN-γ SP cell subsets, and subsequently, gene expression analyses were performed in each of these cell subsets.

To this end, it was necessary to establish a sorting protocol that was compatible with RNA isolation. The common proceeding to label cytokine producing cells is based on an intracellular cell staining that requires cell fixation with formaldehyde. However, fixation of cells can drastically alter the quality of mRNA (von Smolinski et al., 2005). In order to circumvent this, isolation of living cytokine producing cells for subsequent gene expression analysis was necessary. For this purpose, a combined IFN-γ and IL-2 secretion assay without formaldehyde fixation of cells was established in this work. This method enabled labeling and subsequent separation of four living cytokine producing subpopulations of memory CD4 T cells, i. e DN, IL-2 SP, DP and IFN-γ SP cells. In this way, high quality (RIN ≥ 8.9) total RNA was isolated from these separated cell subsets and a global gene expression analysis was performed with Affymetrix-based mouse gene chips.

Analysis of gene expression data is principally based on comparisons of gene expression profiles between given samples. For this comparison, different methods can be used in order to calculate similarities and dissimilarities of samples, which are being compared. In this work, Principal Coordinates Analysis (PCoA) and Pearson Correlation Coefficient (PCC) were used, since these well-established mathematical techniques provide an optimal two-dimensional visual representation of samples.

PCoA, a method of multidimensional scaling, generates axes to summarize the variability in a data set. Each sample has coordinates along these axes, which help to identify the sample on the ordination plot (Gower, 1966; Legendre and Legendre, 1998). PCoA enables to explore and visualize similarities and dissimilarities of the overall gene expression profiles between the four sample groups (Figure 26). Individual samples (or replicates) are represented as single dots and can be compared to each other, according to the distances of their distribution on the 2D graphic of the PCoA, where close distances between samples indicate higher similarity and further distances higher dissimilarity in their global gene expression profiles.

PCoA analysis showed a clear separation of the four cell subsets (DN, IL-2 SP, DP and IFN-γ SP), demonstrating subset-specific characteristics of gene expression profiles. At the same time, replicates within biological groups appeared close to each other, indicating robustness of the gene expression profiles within the groups (Figure 26).

Most prominent distinctness was observed in gene expression of DN cells. In contrast, less difference was seen in the comparison of DP cells to IL-2 SP cells, and DP cells to IFN-γ SP cells (Figure 26).

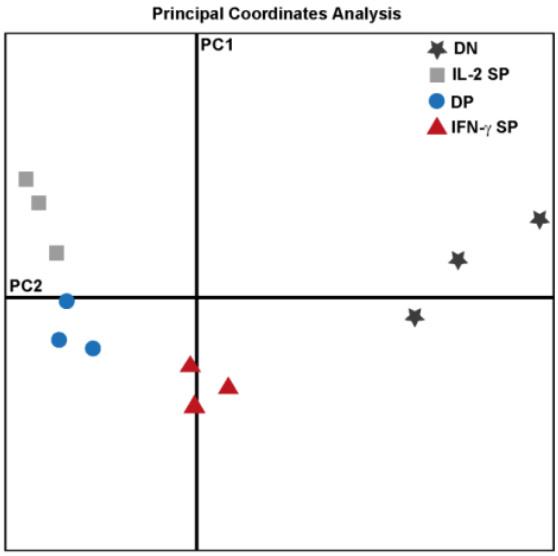

Figure 26. Principal Coordinates Analysis (PCoA) of gene expression data.
Gene expression data were gained using Affymetrix-based mouse gene chips and R software from DN, IL-2 SP, DP, IFN-γ SP cell subsets, isolated from PMA and Ionomycin stimulated memory CD4 T cells of diseased NZBxW lupus-prone mice. The 2D graphic of PCoA illustrates the distribution of four sample groups: DN (dark grey asterisk), IL-2 SP (light grey square), DP (blue circle) and IFN-γ SP (red triangle). Each spot within the groups represents one individual biological replicate. N=3.

Another commonly used method to investigate the similarity between gene expression profiles is the Pearson Correlation Coefficient (PCC) (Eisen et al., 1998), where the highest similarity (or identity) is indicated by a PCC value of 1.0. For current data, the range of PCC values of all samples was between 0.98 and 1.0 (Figure 27). Within the comparisons of the biological groups, the highest dissimilarity in overall gene expression was observed between DN vs. IL-2 SP and to a lesser extent DN vs. DP cells. In contrast, the highest similarity was observed between DP vs. IL-2 SP cells and DP vs. IFN-γ SP cells.

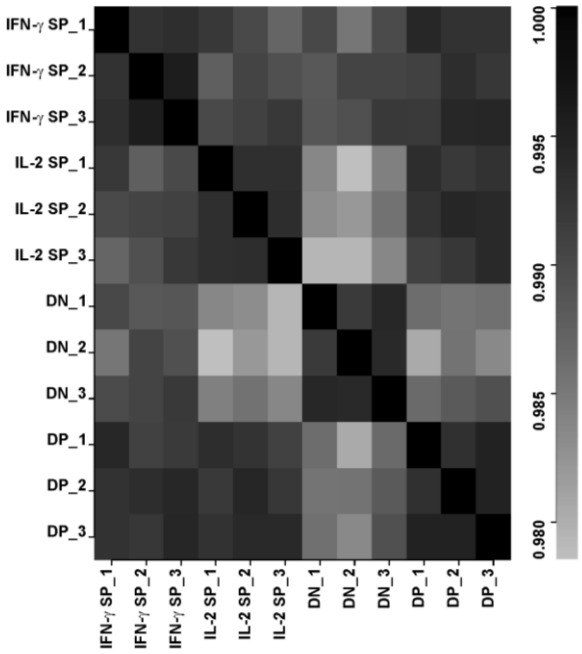

Figure 27. Pearson Correlation Coefficient (PCC) analysis of gene expression data.
Gene expression data were gained using Affymetrix-based mouse gene chips and R software from DN, IL-2 SP, DP, IFN-γ SP cell subsets, isolated from PMA and Ionomycin stimulated memory CD4 T cells of diseased NZBxW lupus-prone mice. The heat map shows correlations between all possible sample comparisons. Intensity of correlation is shown in a color-coded manner from black (PCC value of 1.0, representing identity) to light grey (PCC value of 0.98, representing the lowest observed correlation). Numbers next to sample names (1, 2, 3) represent the three independent biological replicates. N=3.

Taken together, PCoA and PCC analyses showed that all three replicates in each group clustered together, indicating good quality of the samples. DN cells showed most different gene expression profile, compared to all other sample groups. Gene expression profile of DP cells was close to that of IL-2 SP and IFN-γ SP cells. This could be explained by the fact that DP cells were able to produce both cytokines, IL-2 and IFN-γ, whereas DN cells did not produce any of these cytokines and may represent another state of activation or a more distinct subgroup of memory CD4 T cells.

3.3.2. Different gene expression between IFN-γ SP and DP cells

After analyses of similarities and dissimilarities of the overall gene expression profiles between samples, the next aim of this work was to characterize significant alterations in gene expression of single genes between the four memory CD4 T cell subsets (DN, IL-2 SP, DP and IFN-γ SP) of

diseased NZBxW lupus-prone mice. These four samples permitted six possible comparisons: (1) IL-2 SP vs. DP, (2) IFN-γ SP vs. DP, (3) DN vs. DP, (4) IFN-γ SP vs. IL-2 SP, (5) IL-2 SP vs. DN and (6) IFN-γ SP vs. DN. Statistical analysis was carried out using Fisher's exact test with the cutoff for p value by 0.05 as a value for differential gene expression.

The results showed that over 2000 genes were significantly ($p<0.05$) differentially expressed between each comparison, including both up- and downregulated genes (Figure 28). In accordance with global gene expression analysis (see 3.3.1.), the highest numbers of differentially expressed genes were seen between the comparisons of IL-2 SP to DN cells (6134 genes), followed by DN to DP cells (5302 genes) and then by IFN-γ SP to IL-2 SP cells (3933 genes); whereas the comparisons of IL-2 SP to DP cells (2101 genes), IFN-γ SP to DN cells (2944) and IFN-γ SP to DP cells (2577 genes) revealed similar numbers of significantly differentially expressed genes (Figure 28).

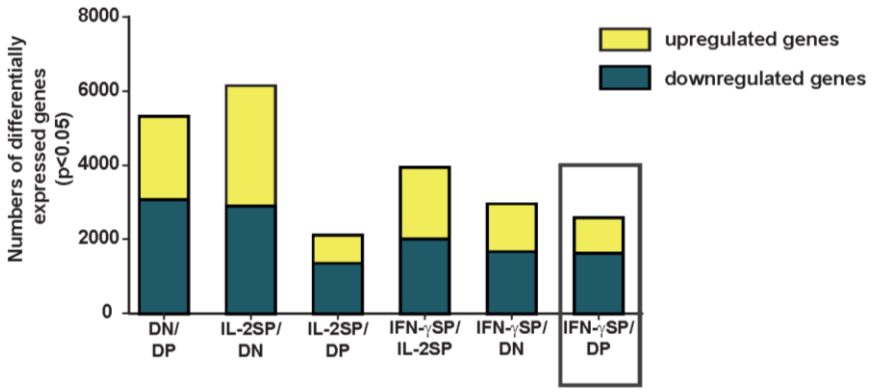

Figure 28. Differentially expressed genes between subsets of memory CD4 T cells.
Gene expression data were gained using Affymetrix-based mouse gene chips and R software from DN, IL-2 SP, DP and IFN-γ SP cell subsets, isolated from PMA and Ionomycin stimulated memory CD4 T cells of diseased NZBxW lupus-prone mice. The bar diagram shows numbers of significantly ($p<0.05$) differentially expressed genes between the six possible comparisons of samples. Significance was calculated using Fisher's exact test. Yellow and dark green bars represent numbers of up- and downregulated genes, respectively. N=3

As next, the focus of this work was put on the gene expression profiles of IFN-γ SP and DP cells in more detail for a number of reasons: First, the main purpose of this work was to follow increased IFN-γ production by memory CD4 T cells in diseased NZBxW lupus-prone mice and characterize altered features of IFN-γ producing memory CD4 T cells. Second, particularly in this comparison (IFN-γ SP vs. DP) small differences in their gene expression profiles were expected,

since both subsets were able to produce IFN-γ. Third, DP cells represent a fully immunoactive memory T cell subset and produce not only IFN-γ, but also other cytokines, e.g. IL-2 and TNF-α (as shown later).

Differentially expressed genes (in IFN-γ SP vs. DP cells; $p<0.05$) that represent surface molecules, secreted proteins or transcription factors were selected (Figure 29). This comparison identified that IFN-γ SP cells were characterized by

- increased expression of inflammatory chemokine receptors (*Cxcr6, Cxcr4, Ccr8, Ccr5, Ccr2, Ccr9*),
- increased expression of cytokine receptors (*Il12rb2, Ifngr1, Il21r, Il18r1*),
- increased expression of co-inhibitory receptors (*Lag3, Ctla2a, Cd80, Ctla4*),
- increased expression of co-stimulatory receptors (*Cd27, Cd40lg*),
- increased expression of granzymes (*Gzma, Gzmb*),
- specific pattern of transcription factors, e.g. increased expression of *Stat1, Satb1, Irf1, Irf9, Smad2,* but decreased expression of *Gata3* and *Myc*,
- decreased cytokine expression (*Il9, Tnf, Ifng, Il18, Il17a, Il17f, Il2, Il5, Il3)*, except increased expression of *Il10.*

Taken together, memory CD4 T cell subsets (DN, IL-2 SP, DP and IFN-γ SP) of diseased NZBxW lupus-prone mice showed more or less distinct gene expression profiles. Moreover, IFN-γ SP cells, which lack the ability to produce IL-2 were characterized by an activated phenotype, displaying increased expression of a range of pro-inflammatory factors, such as chemokine and cytokine receptors, co-inhibitory and co-stimulatory receptors, pro-apoptotic genes, a specific pattern of transcription factors, responsible for lineage specificity and regulation of inflammation and additionally decreased expression of immunomodulatory cytokines.

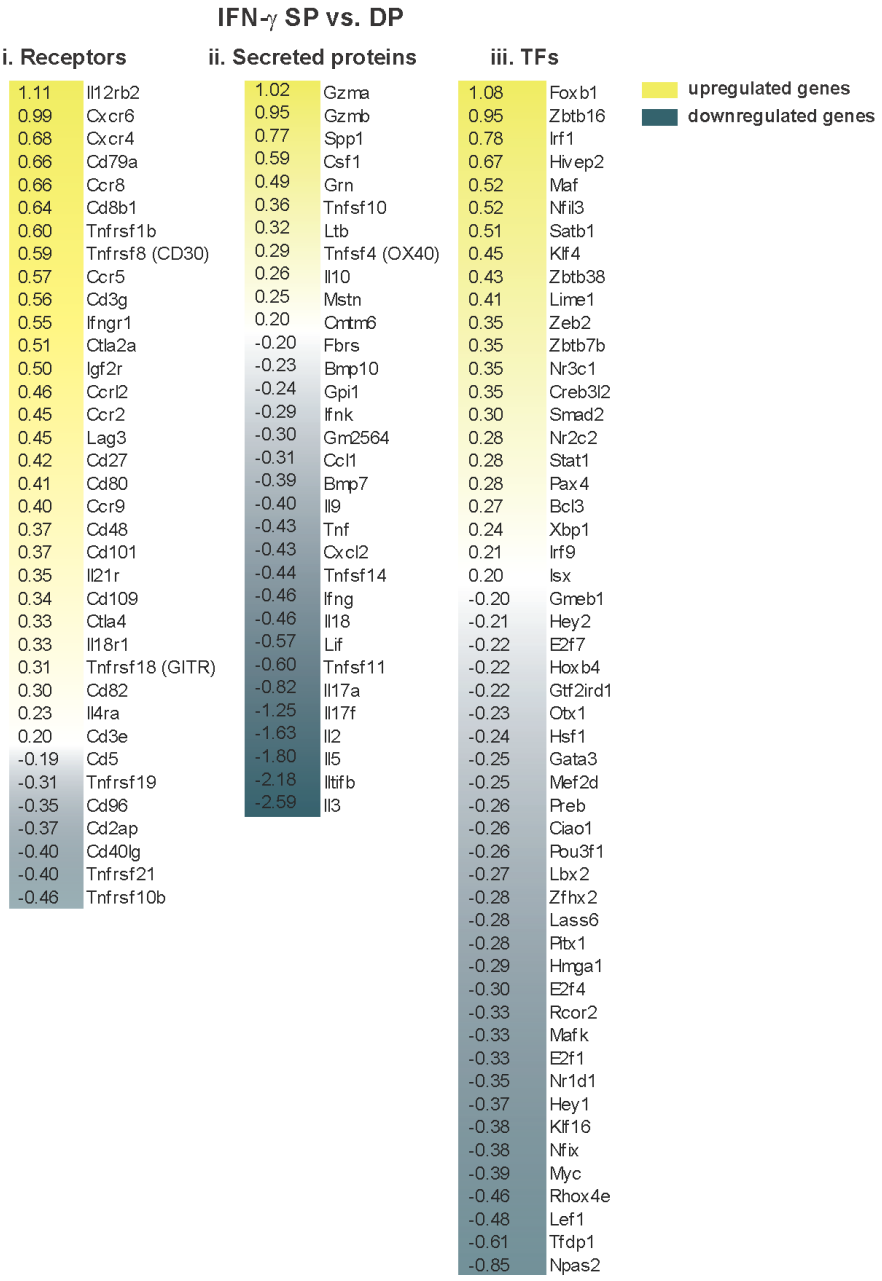

Figure 29. Differentially expressed genes in IFN-γ SP vs. DP cells.
Gene expression data were gained using Affymetrix-based mouse gene chips and R software. Heat maps illustrate differentially expressed genes between IFN-γ SP and DP cells, obtained from over-represented GO terms. Significance was determined by Fisher's exact test; $p<0.05$. In the first column (i) receptor genes are listed, in the second (ii) – secreted proteins and in the third (iii) – transcription factors (TFs). Up- (yellow) and downregulated genes (dark green) were determined from log2 fold change values.

3.4. Analysis of microarray data on protein level

3.4.1. Equal expression of housekeeping genes in all samples

Housekeeping genes are called genes, which are constitutively expressed in all cells and maintain basic cellular functions. Under normal conditions, housekeeping genes are expressed at relatively constant levels in all cells, independent of their type, activation state and specific function (Koonin, 2000).

Taking in account that qRT-PCR is a very sensitive method, factors like pipetting mistakes, inefficient DNA synthesis and inexact quantification can lead to significant differences. To minimize the variability, housekeeping genes are widely used as internal controls for experiments with RNA expression analysis, where an adequate housekeeping gene serves as a reference point for normalization of mRNA levels between distinct samples (Thellin et al., 1999).

However, many of those commonly used housekeeping genes show large variability in their expression, dependent on certain circumstances (Deindl et al., 2002; Glare et al., 2002; Hamalainen et al., 2001; Zhong and Simons, 1999).

Eisenberg et al. published a list of housekeeping genes from available databases of microarray results. Thereby, the housekeeping genes were constitutively expressed in all tissues (Eisenberg and Levanon, 2003).

To further analyze the microarray data of this work via qRT-PCR-based mRNA expression analysis, an adequate housekeeping gene was determined. To this end, a set of 20 housekeeping genes from the list, suggested by Eisenberg et al. was selected and tested for uniform expression between cell samples. The results revealed no significant differences with regard to the expression of all housekeeping genes tested between four distinct samples (DN, IL-2 SP, DP and IFN-γ SP) (data not shown). As an example, expression of *Ribosomal protein S18 (Rps18)* is shown here (Figure 30), since *Rps18* was used as an internal control for normalization of mRNA expression throughout all following qRT-PCR analyses.

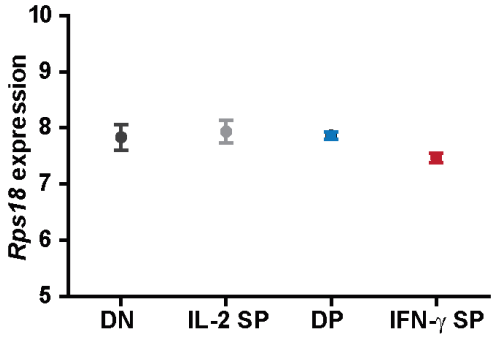

Figure 30. Uniform expression of *Rps18* housekeeping gene.
Gene expression data were gained using Affymetrix-based mouse gene chips and R software from DN, IL-2 SP, DP and IFN-γ SP cell subsets, isolated from PMA and Ionomycin stimulated memory CD4 T cells of diseased NZBxW lupus-prone mice. The diagram shows RNA expression levels of *Ribosomal protein S18 (Rps18)* housekeeping gene. The raw expression values were background corrected, Log2 transformed and normalized by RMA method. Significance was determined by t-test, $p>0.05$. N=3.

3.4.2. Increased expression of co-inhibitory receptors in IFN-γ SP vs. DP cells

One remarkable result of the current microarray-based gene expression analysis is that IFN-γ SP cells, isolated from memory CD4 T cells of diseased NZBxW lupus-prone mice were characterized by an enhanced expression of many inflammatory molecules, such as inflammatory chemokines, co-inhibitory and co-stimulatory receptors, apoptotic markers, a specific pattern of transcription factors and decreased expression of immunomodulatory effector cytokines (see 3.3.2.).

In order to further investigate these features, the expression of several markers relevant for chronic inflammation were determined on RNA and protein levels using qRT-PCR and flow cytometry, respectively. Thereby, the focus was set to the comparison between IFN-γ SP and DP cells, as both cell subsets showed most similar global gene expression (see 3.3.), and the hypothesis of this work was that IFN-γ SP cells may represent a disease-associated analogon to DP cells.

First, two important co-inhibitory receptors, lymphocyte activation protein 3 (LAG-3) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), that are relevant for chronic activation were analyzed on RNA and protein levels. The above presented microarray data showed that the expression of *Lag3* and *Ctla4* were significantly increased by IFN-γ SP cells compared to DP cells. Indeed, their increased expression could be verified on RNA level using qRT-PCR.

Additionally, flow cytometric analysis proofed significantly enhanced protein levels of both receptors (Figure 31and Figure 32).

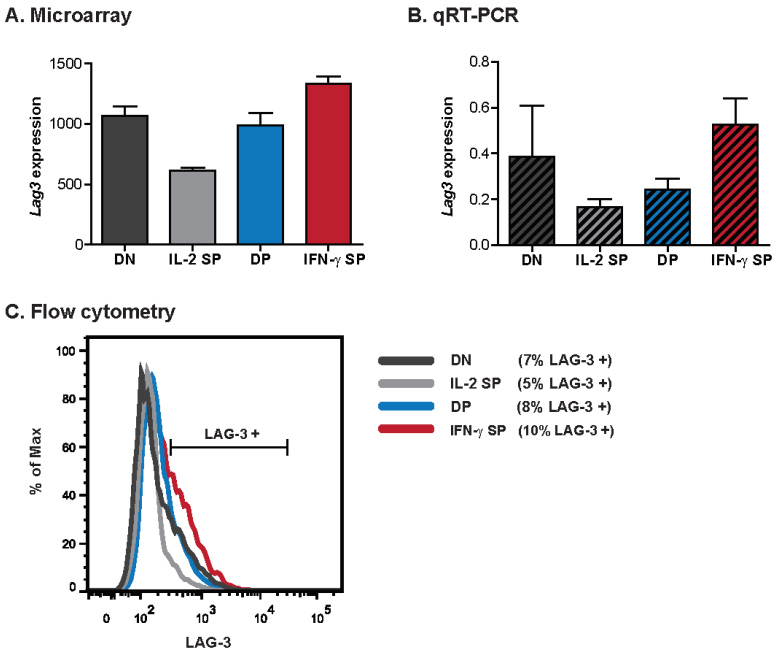

Figure 31. Increased expression of LAG-3 by IFN-γ SP cells of diseased NZBxW lupus-prone mice. Memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were stimulated for 5 h with PMA and Ionomycin and sorted via cytokine expression assay into DN, IL-2 SP, DP and IFN-γ SP cell subsets. In these cell subsets, expression levels of *Lymphocyte activation gene 3 (Lag3)* were measured by (A) microarray analysis and (B) qRT-PCR. Raw microarray data were background corrected and normalized by RMA method. mRNA expression levels of *Lag3* by qRT-PCR were normalized to those of *Rps18* reference gene. (C) For flow cytometric analysis, surface LAG-3 and intracellular cytokine staining of stimulated memory CD4 T cells was used. N=3-6.

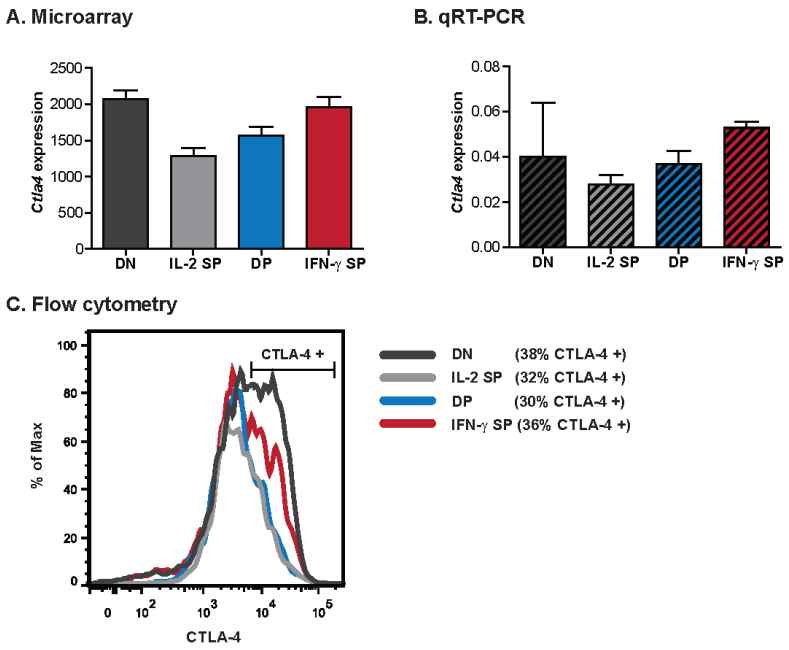

Figure 32. Increased expression of CTLA-4 by IFN-γ SP cells of diseased NZBxW lupus-prone mice. Memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were stimulated for 5 h with PMA and Ionomycin and sorted via cytokine expression assay into DN, IL-2 SP, DP and IFN-γ SP cell subsets. In these cell subsets, expression levels of *Cytotoxic T lymphocyte-associated protein 4 (Ctla4)* were measured by (A) microarray analysis and (B) qRT-PCR. Raw microarray data were background corrected and normalized by RMA method. mRNA expression levels of *Ctla4* by qRT-PCR were normalized to those of *Rps18* reference gene. (C) For flow cytometric analysis, surface CTLA-4 and intracellular cytokine staining of stimulated memory CD4 T cells was used. N=3-6.

In fact, it is known that chronically activated T_H1 cells are characterized by increased expression of co-inhibitory receptors, decreased expression of immunomodulatory cytokines, increased apoptosis and decreased proliferation potentials (Muñoz et al., 2010). In accordance, high expression of LAG-3 and CTLA-4 co-inhibitory receptors may represent features of at least partly chronically activated phenotype of IFN-γ SP cells of diseased NZBxW lupus-prone mice.

3.4.3. Altered cytokine expression in IFN-γ SP vs. DP cells

Next to high expression levels of co-inhibitory receptors, another remarkable feature of chronically activated phenotype is low expression of immunomodulatory cytokines (Muñoz et al., 2010). Therefore, expression of effector cytokines by IFN-γ SP cells was analyzed on RNA and protein levels using qRT-PCR and flow cytometry, respectively.

Microarray-based gene expression analyses of the current work indicated that expression of *Tumor necrosis factor α (Tnf)* was significantly decreased and the expression of *Interleukin 10 (Il10)* was significantly increased in IFN-γ SP cells, when compared to DP cells. This could further be validated using qRT-PCR. In accordance with results on RNA level, flow cytometry showed that also the protein levels of TNF-α were significantly decreased (Figure 33) and the protein levels of IL-10 were significantly increased (Figure 34) in IFN-γ SP cells compared to DP cells.

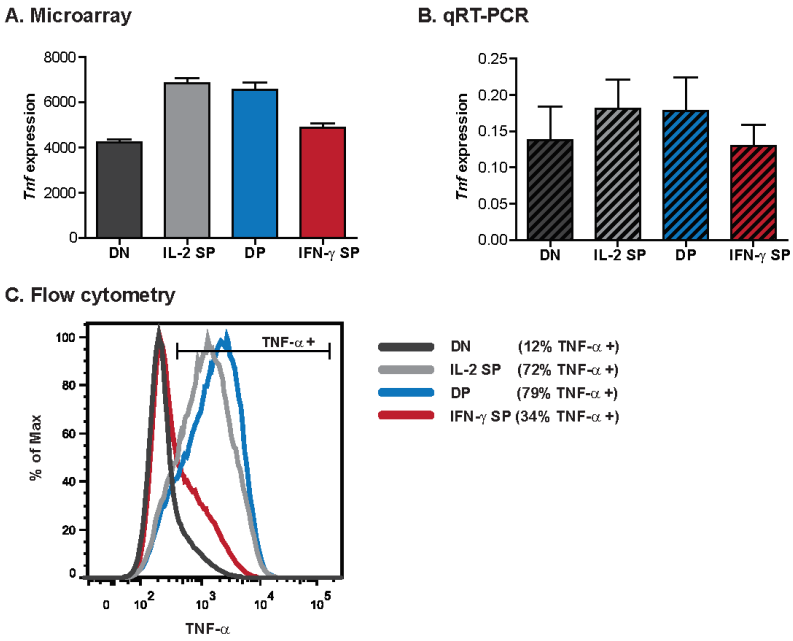

Figure 33. Decreased expression of TNF-α by IFN-γ SP cells of diseased NZBxW lupus-prone mice. Memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were stimulated for 5 h with PMA and Ionomycin and sorted via cytokine expression assay into DN, IL-2 SP, DP and IFN-γ SP cell subsets. In these cell subsets, expression levels of *Tumor necrosis factor α (Tnf)* were measured by (A) microarray analysis and (B) qRT-PCR. Raw microarray data were background corrected and normalized by RMA method. mRNA expression levels of *Tnf* by qRT-PCR were normalized to those of *Rps18* reference gene. (C) For flow cytometric analysis, intracellular cytokine staining of stimulated memory CD4 T cells was used. N=3-6.

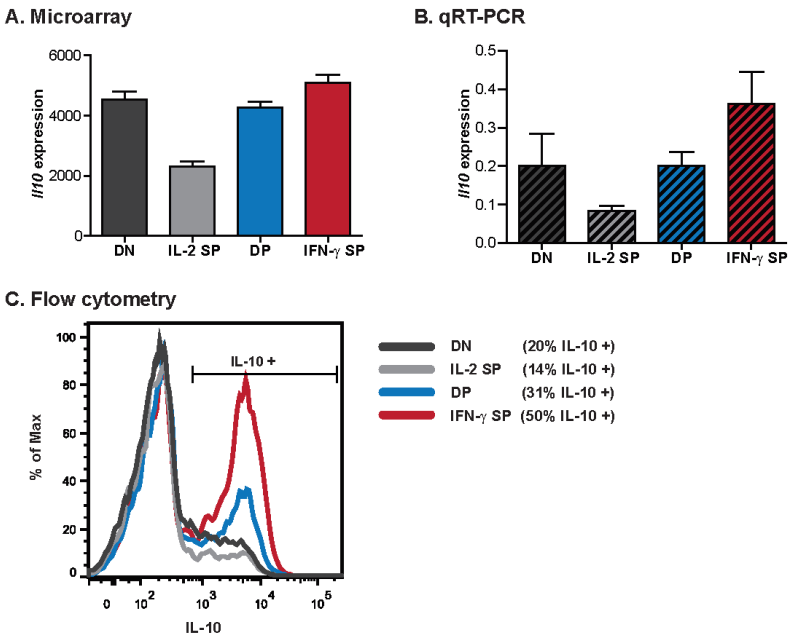

Figure 34. Increased expression of IL-10 by IFN-γ SP cells of diseased NZBxW lupus-prone mice. Memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were stimulated for 5 h with PMA and Ionomycin and sorted via cytokine expression assay into DN, IL-2 SP, DP and IFN-γ SP cell subsets. In these cell subsets, expression levels of *Interleukin 10 (Il10)* were measured by (A) microarray analysis and (B) qRT-PCR. Raw microarray data were background corrected and normalized by RMA method. mRNA expression levels of *Il10* by qRT-PCR were normalized to those of *Rps18* reference gene. (C) For flow cytometric analysis, intracellular cytokine staining of stimulated memory CD4 T cells was used. N=3-6.

To date, the exact functions of TNF-α and IL-10 in SLE have not been clarified yet. However, Jacob et al. showed that decreased expression of TNF-α impaired the course of SLE in NZBxW lupus-prone mice, suggesting that TNF-α might play an immunomodulatory role in this mouse model (Jacob and McDevitt, 1988). Increased expression of IL-10 was shown to positively correlate with disease activity in SLE patients and mice, suggesting its pro-inflammatory role in SLE (Houssiau et al., 1995; Llorente et al., 1995).

Thus, decreased expression of immunomodulatory cytokines, such as TNF-α and IL-2, and increased expression of pro-inflammatory cytokines, such as IL-10, might represent further features of chronically activated phenotype of IFN-γ SP cells of diseased NZBxW lupus-prone mice.

3.4.4. Altered expression of apoptosis-related genes in IFN-γ SP vs. DP cells

It is known that next to increased expression of co-inhibitory receptors and decreased expression of immunomodulatory cytokines, chronically activated T_H1 cells are characterized by increased apoptosis and decreased proliferation capacities (Muñoz et al., 2010). Therefore, expression of apoptosis-related genes were analyzed in IFN-γ SP cells of diseased NZBxW lupus-prone mice.

Microarray analyses of this work showed that the expression of granzymes was increased in IFN-γ SP cells, when compared to DP cells. Granzime B is known to induce apoptosis of target cells (Lord et al., 2003). Expression of this member of the granzyme family was further analyzed on RNA and protein levels using qRT-PCR and flow cytometry, respectively.

In accordance to microarray-based gene expression analyses, increased expression of granzyme B was observed in IFN-γ SP cells on RNA and protein levels using qRT-PCR and flow cytometry, respectively (Figure 35).

Increased granzyme B expression might suggest elevated damaging features of IFN-γ SP cells in diseased NZBxW lupus-prone mice.

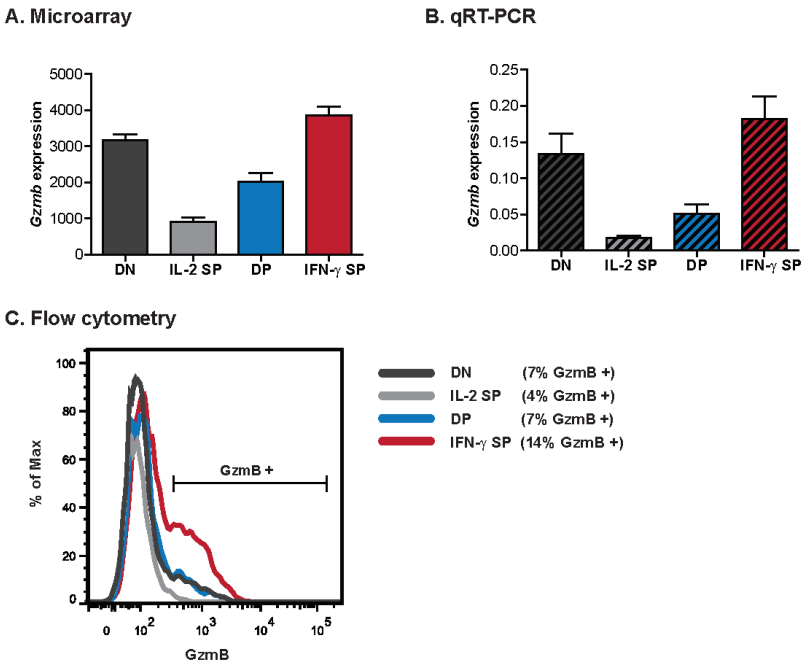

Figure 35. Increased expression of GzmB by IFN-γ SP cells of diseased NZBxW lupus-prone mice. Memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were stimulated for 5 h with PMA and Ionomycin and sorted via cytokine expression assay into DN, IL-2 SP, DP and IFN-γ SP cell subsets. In these cell subsets, expression levels of *Granzyme B (Gzmb)* were measured by (A) microarray analysis and (B) qRT-PCR. Raw microarray data were background corrected and normalized by RMA method. mRNA expression levels of *Gzmb* by qRT-PCR were normalized to those of *Rps18* reference gene. (C) For flow cytometric analysis, surface GzmB and intracellular cytokine staining of stimulated memory CD4 T cells was used. N=3-6.

Next to granzymes, expression of another apoptosis-related gene, *Death associated protein-like 1 (Dapl1)* was found to be within the top 10 of differentially expressed genes in current microarray dataset. In contrast to *Gzmb*, expression of *Dapl1* was significantly decreased in IFN-γ SP cells of diseased NZBxW lupus-prone mice, when compared to DP cells. This could be validated on RNA levels using qRT-PCR analysis (Figure 36). However, due to the unavailability of an adequate antibody for flow cytometric analysis, expression of DAPL-1 could not be investigated on protein level.

The function of DAPL-1 is relatively unknown. The protein is believed to act as a positive mediator of programmed cell death upon induction by IFN-γ (Deiss et al., 1995) and acts as a cell proliferation receptor in retinal pigment epithelial cells (Ma et al., 2017). In this context, diminished DAPL-1 expression by IFN-γ SP cells of diseased NZBxW lupus-prone mice might lead to an increase in cell proliferation.

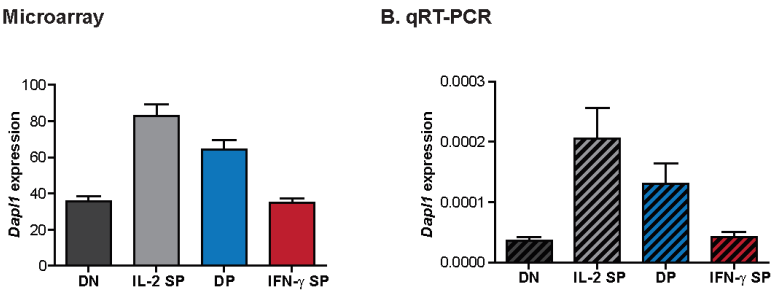

Figure 36. Decreased expression of *Dapl1* by IFN-γ SP cells of diseased NZBxW lupus-prone mice. Expression levels of *Death associated protein-like 1 (Dapl1)* were measured by (A) microarray analysis and (B) qRT-PCR in DN, IL-2 SP, DP and IFN-γ SP subsets of memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice and stimulated for 5 h with PMA and Ionomycin. Raw microarray data were background corrected and normalized by RMA method. mRNA expression levels of *Dapl1* by qRT-PCR were normalized to those of *Rps18* reference gene. N=3-6

3.4.5. Altered expression of transcription factors in IFN-γ SP vs. DP cells

The composition and activation state of the expressed transcription factor repertoire are known to play a crucial role for the lineage specificity and functionality of cells. Therefore, the expression of two crucial transcription factors of T cell identity were analyzed, namely Interferon regulatory factor 1 (IRF-1) and Special AT-rich sequence binding protein 1 (SATB-1), that were shown to contribute to the pathogenesis of SLE in mouse models (Reilly et al., 2006; Thibault et al., 2008). Both *Irf1* and *Satb1* appeared to be significantly increased in IFN-γ SP cells of diseased NZBxW lupus-prone mice, as assessed by current microarray analysis.

As expected, qRT-PCR analyses were in accordance with microarray data, demonstrating that the expression of both *Satb1* and *Irf1* were increased in IFN-γ SP cells, compared to DP cells of diseased NZBxW lupus-prone mice (Figure 37 and Figure 38, respectively). Analysis of these transcription factors by flow cytometry, however, was not possible due to unavailability of adequate antibodies.

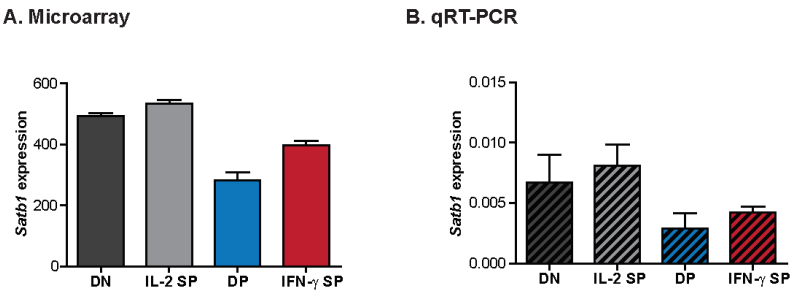

Figure 37. Increased expression of *Satb1* by IFN-γ SP cells of diseased NZBxW lupus-prone mice.
Expression levels of *Special AT-rich sequence binding protein 1 (Satb1)* were measured by (A) microarray analysis and (B) qRT-PCR in DN, IL-2 SP, DP and IFN-γ SP subsets of memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice and stimulated for 5 h with PMA and Ionomycin. Raw microarray data were background corrected and normalized by RMA method. mRNA expression levels of *Satb1* by qRT-PCR were normalized to those of *Rps18* reference gene. N=3-6

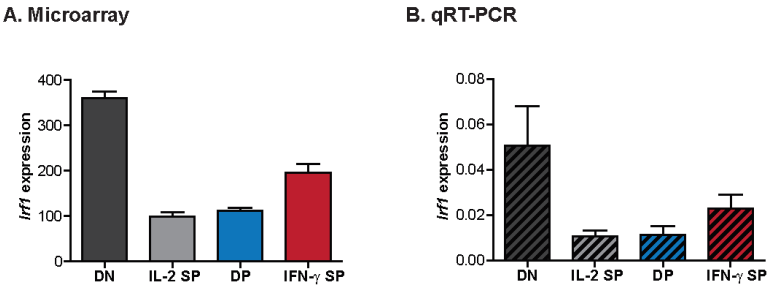

Figure 38 Increased expression of *Irf1* by IFN-γ SP cells of diseased NZBxW lupus-prone mice.
Expression levels of *Interferon regulatory factor 1 (Irf1)* were measured by (A) microarray analysis and (B) qRT-PCR in DN, IL-2 SP, DP and IFN-γ SP subsets of memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice and stimulated for 5 h with PMA and Ionomycin. Raw microarray data were background corrected and normalized by RMA method. mRNA expression levels of *Irf1* by qRT-PCR were normalized to those of *Rps18* reference gene. N=3-6

Taken together, despite the similarity of the global gene expression pattern of IFN-γ SP and DP cells (Figure 26), directed expression analysis of selected genes showed that IFN-γ SP cells were characterized by altered gene expression, when compared to their DP counterparts in features, including co-inhibitory surface molecules, cytokine, apoptosis-related genes and transcription factors. Remarkably, the alterations of these genes were in comparable direction as seen for chronically activated T_H1 cells (Muñoz et al., 2010), as IFN-γ SP cells exhibited increased expression of co-inhibitory surface molecules (LAG-3, CTLA-4), decreased expression of effector cytokines (TNF-α, IL-2), but increased expression of IL-10, as well as a specific pattern of apoptotic genes, such as increased expression of granzyme B and decreased expression of

DAPL-1; furthermore, they exhibited increased expression of T_H1 specific transcription factors (STAB-1, IRF-1). Thus, these altered features might point to a chronically activated phenotype of IFN-γ SP cells of diseased NZBxW lupus-prone mice.

3.5. Overlap of genes between IFN-γ SP cells in SLE and exhausted, effector and memory T cells in viral infection

As shown in this work, the IFN-γ SP cell subset of memory CD4 T cells of diseased NZBxW lupus-prone mice was characterized by reduced production of effector cytokines and increased expression of co-inhibitory receptors. A state of T cell dysfunction with the progressive loss of effector functions and increased expression of co-inhibitory receptors occurs usually during chronic infections and cancers and is commonly known as T cell exhaustion (Wherry, 2011). On this account, microarray-based gene expression data of current work were compared with the published gene list of exhausted T cells (Crawford et al., 2014).

T cell exhaustion was described more than twenty years ago and was investigated preferentially in CD8 T cells (Zajac et al., 1998). In 2014, Crawford et al. published a microarray dataset of CD4 T cell exhaustion in a mouse model of chronic infection by lymphocytic choriomeningitis virus (LCMV) using genome-wide transcriptional profiling. In the experimental setup of Crawford et al., two distinct strains of LCMV were used to induce either an acute (Armstrong virus) or a chronic infection (Clone 13), whereby T cell exhaustion occurs only during chronic infection (Wherry et al., 2003). This setup allowed to define three subsets of CD4 T cells: (1) effector CD4 T cells, *ex vivo* isolated on day 8 after infection of mice with Armstrong virus; (2) memory CD4 T cells, *ex vivo* isolated on day 30 after infection of mice with Armstrong virus, and (3) exhausted CD4 T cells, *ex vivo* isolated on day 30 after infection of mice with Clone13 (Crawford et al., 2014). The study of Crawford et al. revealed significant differences between exhausted and memory CD4 T cells. In detail, exhausted CD4 T cells were characterized by a distinct pattern of co-inhibitory (increased *Btla, Ctla4, Cd200, Lag3, Pdcd1* (PD1)) and co-stimulatory receptors (increased *Cd86, Tnfrsf4* (Ox40), *Icos, Cd27*, but decreased *Cd28*), a unique transcription factor profile (*Fosb, Id2, Batf, Jun, Junb*, increased expression of *Klf6, Eomes, Prdm1* (Blimp1), *Ikzf2* (Helios), *Gata3, Bcl6*, and decreased expression of *Tbx21* (T-bet) and *Tcf7*), specific cytokine pattern (decreased expression of *Ifng, Tnf, Il2*, and increased expression of *Il10 and Il21*) (Crawford et al., 2014).

Since many of these exhaustion-associated signature genes were differentially expressed in IFN-γ SP cell subset of memory CD4 T cells of diseased NZBxW lupus-prone mice, namely increased expression of *Ctla4, Lag3, Cd86, Tnfrsf4* (Ox40), *Cd27, Il10* and decreased expression of *Ifng, Tnf and Il2*; microarray-based gene expression data of current work were compared with the gene lists of exhausted, memory and effector CD4 T cells of mice with viral infections, published by Crawford et al.

For the comparison of the two datasets, a custom-created program based on R software was used. This analysis method allowed assessment of the randomness of the observed overlap between the two datasets. Significance was calculated using one-tailed version of Fisher's exact test.

The comparison revealed a significant overlap of genes, upregulated in IFN-γ SP vs. DP cells of diseased NZBxW lupus-prone mice with genes, upregulated in exhausted vs. effector CD4 T cells of mice with viral infections (137 genes, $p<0.0001$) (Figure 39A). In contrast, the overlap of genes, upregulated in IFN-γ SP vs. DP cells of diseased NZBxW lupus-prone mice was not significant with genes, upregulated in memory vs. effector CD4 T cells of mice with viral infections (62 genes, $p>0.05$) (Figure 39A).

The overlap of genes, downregulated in IFN-γ SP vs. DP cells of NZBxW lupus-prone mice was significant for both comparisons: Exhausted vs. effector (202 genes, $p<0.0001$) and memory vs. effector CD4 T cells of mice with viral infections (171 genes, $p=0.005$) (Figure 39B).

In summary, a significant part of genes up- and downregulated in IFN-γ SP cells of diseased NZBxW lupus-prone mice significantly overlapped with genes of exhausted CD4 T cells, generated during chronic viral infection; and only downregulated genes in IFN-γ SP cells significantly overlapped with genes of memory CD4 T cells, generated during acute viral infection. Thus, the pattern of gene upregulation of IFN-γ SP cells of diseased NZBxW lupus-prone mice was more comparable with that of exhausted CD4 T cells and less with memory CD4 T cells, generated during viral infections.

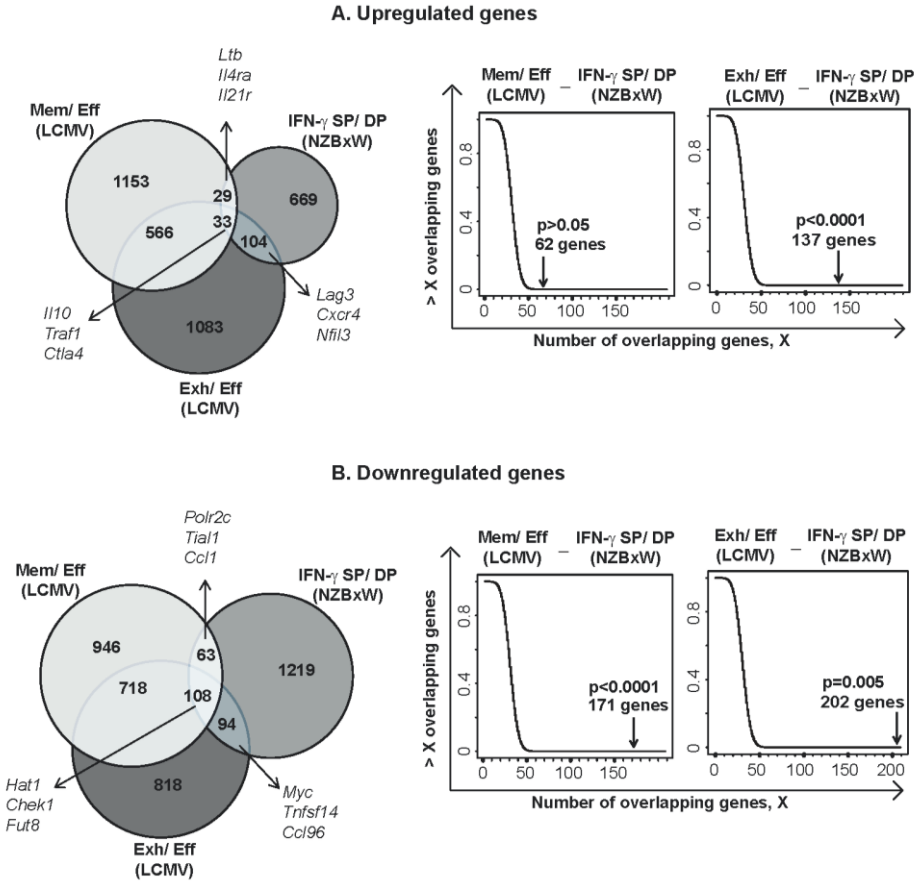

Figure 39. Genes, differentially expressed between IFN-γ SP vs. DP cells of diseased NZBxW lupus-prone mice overlap with genes, differentially expressed between memory vs. effector, and exhausted vs. effector CD4 T cells of mice infected with LCMV.
Panels (A) and (B) show up- and downregulated genes, respectively. On the left side, Venn diagrams are presented with the numbers of overlapping genes. For each overlap, three (most known) example genes are shown. On the right side, significances of the observed overlap are presented. On the x axis the numbers of overlapping genes (X) are depicted. On the y axis the probability of more than X overlapping genes is plotted. Significance was determined using one-tailed version of Fisher's exact test.

Hence, the alterations in gene expression of IFN-γ SP cells might point to an altered functional profile of this particular subset of memory CD4 T cells of diseased NZBxW lupus-prone mice. In order to follow this hypothesis, further functional analyses of memory CD4 T cell subsets were performed.

3.6. Functional properties of IFN-γ SP cells of diseased NZBxW lupus-prone mice

3.6.1. Stable IFN-γ production by memory CD4 T cells upon IFN-γ administration to *in vitro* cultures

In order to follow functional features of IFN-γ SP cells, the stability of cytokine production as well as apoptosis and proliferation potentials of these cells were determined.

In order to investigate the stability of cytokine production, memory CD4 T cells, *ex vivo* isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were stimulated with PMA and Ionomycin for 5 h to induce cytokine production. The cells were divided into three fractions. One fraction of cells was analyzed by flow cytometry using intracellular cytokine staining (1x stimulation, Figure 40A). Another two cell fractions were sorted via IL-2 and IFN-γ cytokine secretion assay into DN, IL-2 SP, DP and IFN-γ SP cells. One part of sorted cell subsets was cultivated for five days *in vitro*. On day five after cultivation, these cells were again stimulated with PMA and Ionomycin for 5 h and cytokine production was determined by intracellular cytokine staining using flow cytometry (2x stimulation, Figure 40B). In parallel, another part of the sorted cell subsets was cultivated in the presence of TCR stimulation by plate-bound anti-CD3/anti-CD28 antibodies for five days *in vitro.* On day five after cultivation, these cells were restimulated with PMA and Ionomycin for 5 h and cytokine production was determined by intracellular cytokine staining using flow cytometry (3x stimulation, Figure 40C).

Mouse recombinant cytokines IL-2, IL-7 and IFN-γ were added to all *in vitro* cultures. IL-2 and IL-7 are required for proliferation and survival of memory CD4 T cells, respectively. IFN-γ was added to the cultures in order to create an equal cytokine milieu for all cell subsets that was more comparable to the *in vivo* situation.

Cultures without TCR stimulation (2x stimulation, Figure 40B) showed that all memory CD4 T cell subsets, except for IL-2 SP cells, recreated a similar profile of cytokine expression, as observed directly after *ex vivo* isolation (1x stimulation, Figure 40A). Namely, DN, DP and IFN-γ SP cells recreated a population with 35-40% of DP cells and 35-40% of IFN-γ SP cells (Figure 40B).

In contrast, in the presence of TCR stimulation (3x stimulation, Figure 40C) all memory CD4 T cell subsets, except for IL-2 SP cells, showed drastically reduced proportions of DP cells, while they still consisted of 35-40% of IFN-γ SP cells (Figure 40C).

However, due to the requirement of large cell numbers for this particular experiment, two experiments were performed, which did not allow for determination of the significance.

Taken together, memory CD4 T cells of diseased NZBxW lupus-prone mice produced considerable amounts of IFN-γ after repetitive stimulations. However, this observation should be interpreted with caution, since IFN-γ was added to the *in vitro* cultures, which in its turn might have influenced the direction of T cell differentiation towards T_H1 cells.

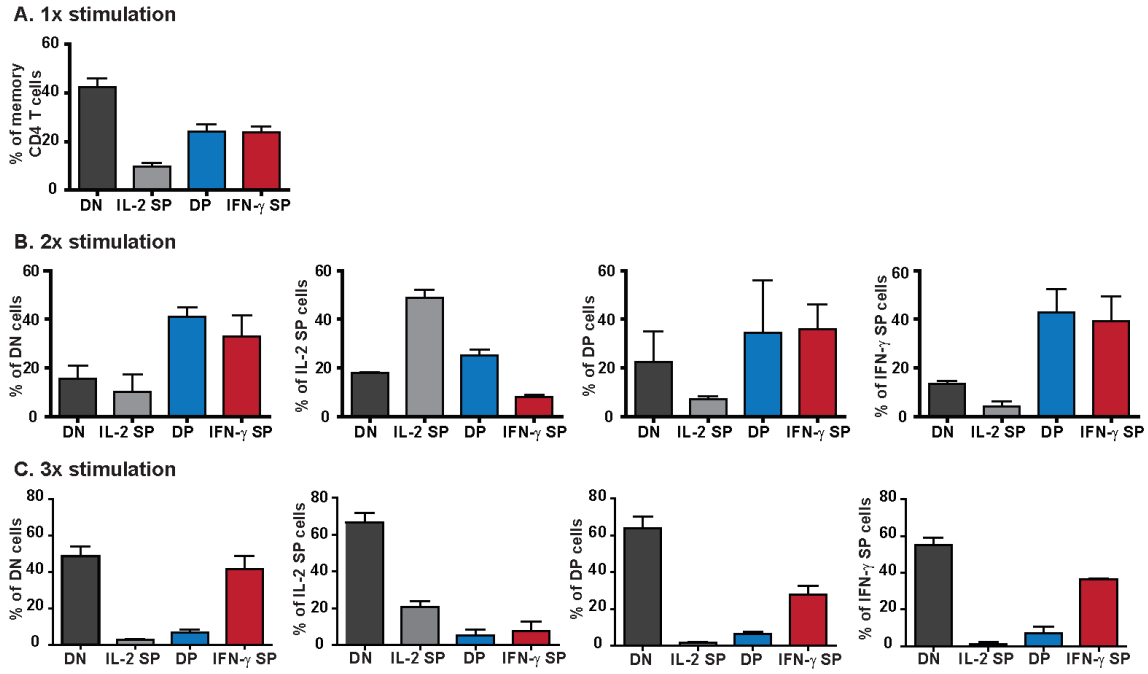

Figure 40. Stable IFN-γ production upon repetitive stimulations under conditions of IFN-γ administration to *in vitro* cultures.
Bar diagrams represent percentages of DN, IL-2 SP, DP and IFN-γ SP cells, (A) *ex vivo* isolated from memory CD4 T cells from spleens and lymph nodes of diseased NZBxW lupus-prone mice and stimulated with PMA and Ionomycin for 5 h (1x stimulation). Stimulated memory CD4 T cells were sorted via IL-2 and IFN-γ secretion assay into DN, IL-2 SP, DP and IFN-γ SP cells, which were *in vitro* cultivated for 5 days (B) without or (C) with presence of TCR stimulation by plate-bound anti-CD3/-CD28 antibodies and restimulated with PMA and Ionomycin on day 5 for 5 h (2x and 3x stimulation, respectively). Mouse recombinant cytokines IL-2, IL-7 and IFN-γ were added to all *in vitro* cultures (B and C). Cells were analyzed by intracellular cytokine staining using flow cytometry. N=2 with a pool of 10 mice per group and experiment.

3.6.2. Decreased proliferation potential of IFN-γ SP cells

To further investigate functional properties of IFN-γ SP cells in SLE, the next aim of this work was to elucidate the proliferative capacity of DN, IL-2 SP, DP and IFN-γ SP cell subsets of memory CD4 T cells of diseased NZBxW lupus-prone mice.

For this purpose, memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were stimulated for 5 h with PMA and Ionomycin in order to induce cytokine production, and sorted via IL-2 and IFN-γ cytokine secretion assay into DN, IL-2 SP, DP and IFN-γ SP cells. Subsequently, these sorted cell subsets were stained with CFSE for proliferation analysis and *in vitro* cultivated for five days. On day five, the proliferation potential was determined by CFSE dilution as well as Ki-67 staining using flow cytometry. Since Ki-67 protein is detected only in active phases of cell cycle, i.e. G_1, S, G_2 and M (dividing cells) and is absent in G_0 phase (resting cells), it is widely used as a proliferation marker (Gerdes et al., 1983).

Ex vivo isolated DN, IL-2 SP, DP and IFN-γ SP cells showed negative Ki-67 staining, indicating that these cells were not proliferating (Figure 41A), whereas on day five after *in vitro* cultivation all cell subsets exhibited proliferative activity, as assessed by positive Ki-67 staining (Figure 41B).

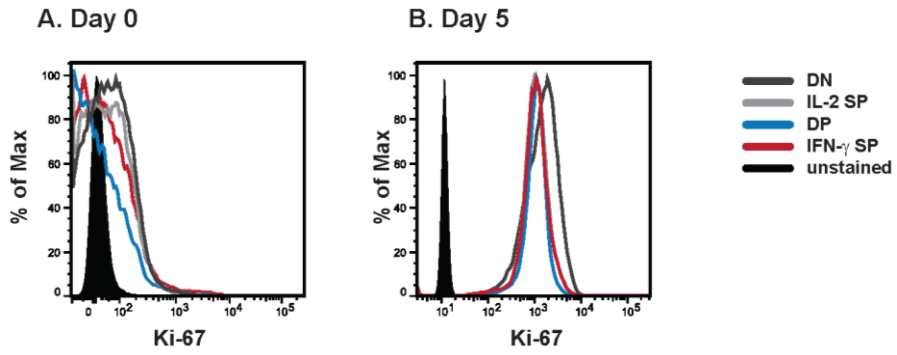

Figure 41. Proliferation of cell subsets of memory CD4 T cells of diseased NZBxW lupus-prone mice defined by Ki-67 staining.
Histograms represent Ki-67 staining of DN (dark grey), IL-2 SP (light grey), DP (blue) and IFN-γ SP (red) cells, (A) *ex vivo* isolated from PMA and Ionomycin stimulated memory CD4 T cells from spleens and lymph nodes of diseased NZBxW lupus-prone mice, or (B) after five days of *in vitro* cultivation. To all cultures mouse recombinant cytokines IL-2, IL-7 and IFN-γ were added. N=2 with a pool of 5 mice per group and experiment.

In order to further define the numbers of cell generations after rapid proliferation, the CFSE assay was used. Therefore, stimulated DN, IL-2 SP, DP and IFN-γ SP cells were stained with CFSE and cultivated for five days *in vitro*. This assay detected at least six generations of proliferation (Figure 42A). Determination of the percentages of proliferating cells and division index revealed that IL-2 SP cells were characterized by the highest proliferative capacity, followed by DP cells and then by IFN-γ SP cells. The lowest proliferative capacity was seen in DN cells (Figure 42B and

C). Since large numbers of cells were required for this particular experiment, only two experiments were performed, which did not allow for determination of the significance.

Nevertheless, IFN-γ SP cells of diseased NZBxW lupus-prone mice tended to show a decreased proliferative capacity during *in vitro* cultures, when compared to DP cells, which might indicate a reduced functionality of IFN-γ SP cells.

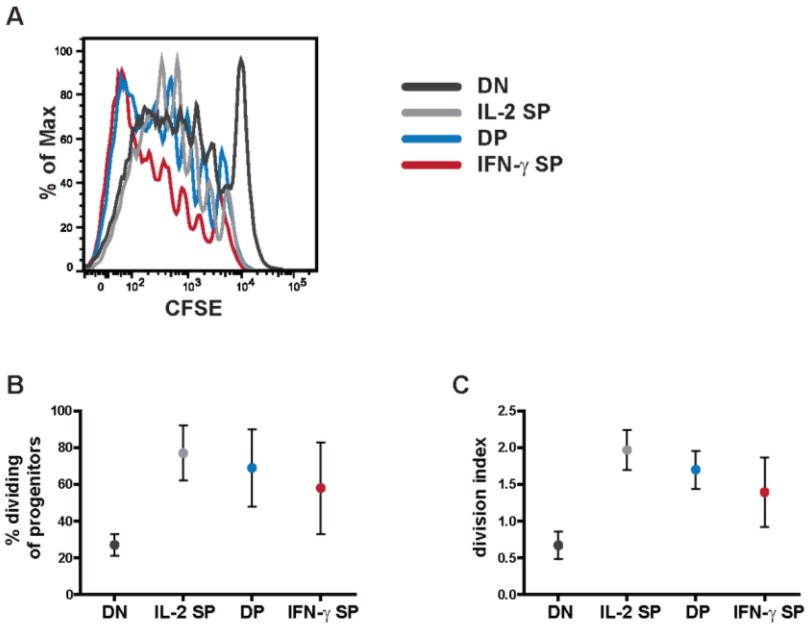

Figure 42. Decreased proliferative capacity of IFN-γ SP vs. DP cells.
Memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice, were stimulated with PMA and Ionomycin and sorted via IL-2 and IFN-γ secretion assay into DN, IL-2 SP, DP and IFN-γ SP cells, which were then stained with CFSE and *in vitro* cultivated for 5 days. (A) Histograms represent CFSE-cell-division of DN, IL-2 SP, DP and IFN-γ SP cells on day 5 of *in vitro* cultivation. (B) Frequencies of dividing progenitors and (C) the division index were calculated using the proliferation tool of FlowJo software. To all *in vitro* cultures mouse recombinant cytokines IL-2, IL-7 and IFN-γ were added. N=2 with a pool of 5 mice per group.

3.6.3. Increased apoptosis potential of IFN-γ SP cells

To further characterize functional properties of DN, IL-2 SP, DP and IFN-γ SP cell subsets of memory CD4 T cells of diseased NZBxW lupus-prone mice, next to the stability of cytokine production and proliferation rates, apoptosis potential of these cells was investigated. For this purpose, memory CD4 T cells were stimulated with PMA and Ionomycin for 5 h and sorted via cytokine secretion assay, according to their capacity of IL-2 and IFN-γ production into DN, IL-2 SP, DP and IFN-γ SP cells. Subsequently, the cells were *in vitro* cultivated for up to five days.

The apoptosis potential was determined using flow cytometry on the first, third and fifth days using the apoptosis marker Annexin V and dead cell stain propidium iodide (PI).

This approach revealed that DN cells tended to show overall the highest frequencies of apoptotic cells, followed by IFN-γ SP, DP and IL-2 SP cells (Figure 43). As shown above, IFN-γ SP cells of diseased NZBxW lupus-prone mice tended to proliferate less than DP cells. In analogy, IFN-γ SP tended to have higher apoptotic capacity than DP cells of these animals, indicating reduced viability of IFN-γ SP cells.

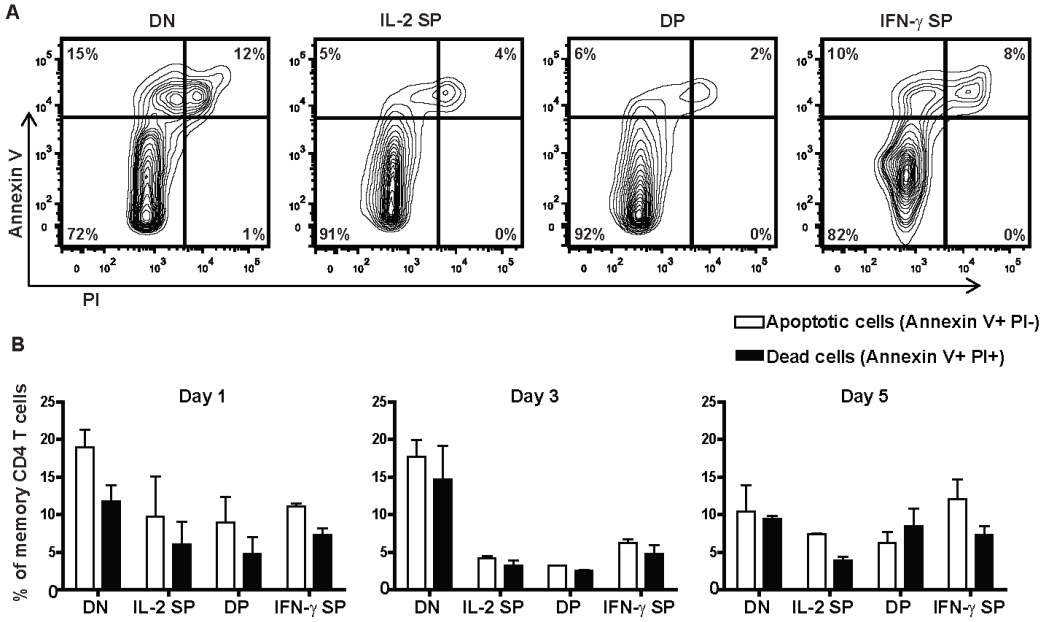

Figure 43. Increased apoptosis potential of IFN-γ SP vs. DP cells.
Memory CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were stimulated with PMA and Ionomycin for 5 h and sorted via IL-2 and IFN-γ secretion assay into DN, IL-2 SP, DP and IFN-γ SP cells, which were then *in vitro* cultivated for up to 5 days. After *in vitro* cultivation, cells were stained with Annexin V and propidium iodide (PI) and analyzed using flow cytometry. (A) Dot plots represent the distribution of apoptotic cells (Annexin V^+PI^-) and dead cells (Annexin V^+PI^+) among DN, IFN-γ SP, DP and IL-2 SP cells on day 5 of *in vitro* cultivation. (B) Bar diagrams represent the frequencies of apoptotic and dead cells among DN, IFN-γ SP, DP and IL-2 SP cells on days 1, 3 and 5 after *in vitro* cultivation. To all *in vitro* cultures mouse recombinant cytokines IL-2, IL-7 and IFN-γ were added. N=2.

3.6.4. Different biological processes of IFN-γ SP vs. DP cells

Microarray-based gene expression analysis of this work revealed that IFN-γ SP cells of diseased NZBxW lupus-prone mice were characterized by an altered gene expression profile, when compared to DP cells. To identify, which biological processes may distinguish IFN-γ SP from DP

cells, the total list of differentially expressed genes between both subsets (IFN-γ SP vs. DP) was analyzed using DAVID, which allows for the detection of over-representative gene ontology (GO) terms.

According to this analysis, biological processes that were associated especially with upregulated genes in IFN-γ SP vs. DP cells included positive regulation of IFN-γ production, positive regulation of cytolysis and cell death as well as negative regulation of molecular functions and transport (Figure 44A).

In contrast, genes that were downregulated in IFN-γ SP vs. DP cells could be linked to biological processes, like RNA metabolic processing and cell cycle process (Figure 44B).

In summary, this analysis helped to identify differences in biological processes between IFN-γ SP vs. DP cell subsets of diseased NZBxW lupus-prone mice. The results suggest rather reduced viability and functionality of IFN-γ SP cells compared to DP cells due to increased regulation of cell death and decreased regulation of mytosis.

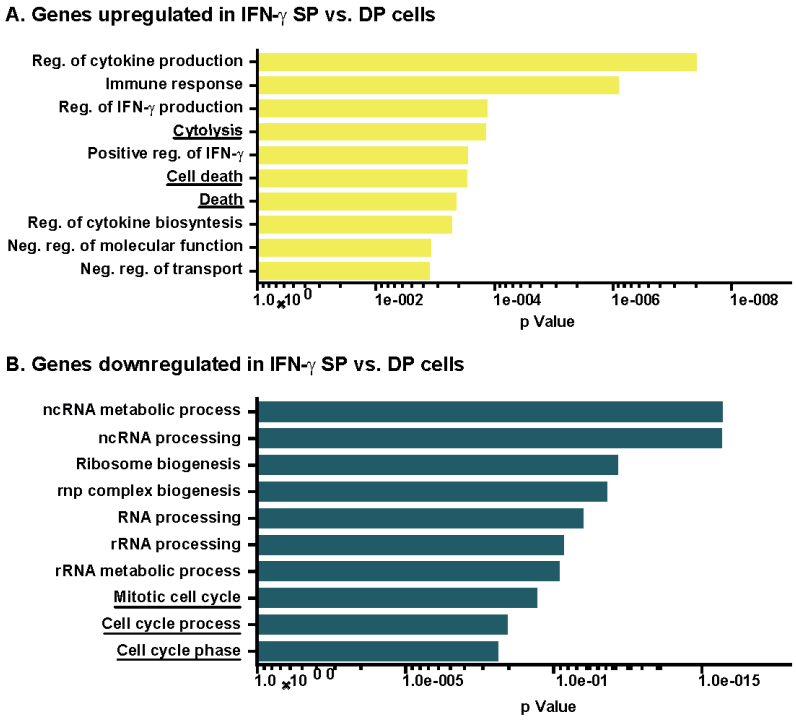

Figure 44. Biological functions affected by altered gene expression in IFN-γ SP vs. DP cells.
Gene expression data were gained using Affymetrix-based mouse gene chips and R software from DN, IL-2 SP, DP and IFN-γ SP cell subsets, isolated from PMA and Ionomycin stimulated memory CD4 T cells of diseased NZBxW lupus-prone mice. Bar diagrams represent the top 10 significantly enriched GO terms referring to biological processes between IFN-γ SP and DP cells. (A) GO terms, that were associated primarily to genes significantly up- (yellow) or (B) downregulated (dark green) in IFN-γ SP vs. DP cells are

presented. Data were analyzed using DAVID software. Significance was determined by Fisher's exact test, $p<0.05$. N=3

3.7. Cytokine expression before and after onset of lupus nephritis

3.7.1. Decreased TNF-α expression by IFN-γ SP cells after onset of LN

Numerous studies demonstrated altered production of various cytokine (Table 1) in SLE and found correlation with disease activity (Smolen et al., 2005). Next to IL-2 and IFN-γ, another cytokine, which was shown to be important for the pathogenesis of SLE is TNF-α (Jacob and McDevitt, 1988). The results of this work revealed that the production of TNF-α was significantly lower in IFN-γ SP cells of diseased NZBxW lupus-prone mice, when compared to DP cells.

Since lupus nephritis (LN) represents most common and probably most sever complication of SLE (Lee et al., 2011), the next aim of this work was to investigate cytokine co-expression in NZBxW lupus-prone mice before and after onset of LN. Disease onset was assessed by age and proteinuria parameters. Young NZBxW lupus-prone mice at the age of 1-2 months before onset of LN displayed no proteinuria (0 g/L), whereas old mice at the age of 5-6 months had massive proteinuria of more than 20 g/L. To analyze cytokine production, memory CD4 T cells, isolated from spleens and lymph nodes of both animal groups were equally stimulated with PMA and Ionomycin and analyzed by flow cytometry using intracellular cytokine staining.

In order to analyze the cytokine combinatorics before and after onset of LN in more detail, a three-parameter visualization tool of data analysis was developed on the basis of R software in the "Signal Transduction" working group at German Rheumatism Research Centre. This tool allows for quantification of three cell parameters at a time. In particular, frequencies of TNF-α, IL-2 and IFN-γ producing memory CD4 T cells were determined using flow cytometric analysis and transformed into R software to create a three dimensional (3D) plot, as described in 2.4.9. In principal, the parameters of IFN-γ and IL-2 production of a given cell were scaled by fluorescent intensity into two-dimensional bins and plotted on the x and y axis, respectively. Each bin was set up to contain at least 20 cells. For each bin, the frequency of TNF-α producers was calculated and plotted in the third dimension using a color-coded heat map (Figure 45).

Three-parameter visualization demonstrated that in both animal groups (before and after onset of LN) IL-2 SP and DP cells produced high frequencies of TNF-α. In contrast, whereas the majority of IFN-γ SP cells before onset of LN was able to produce TNF-α (about 75-100%, Figure 45A, left upper panel), after onset of LN, IFN-γ SP cells lost this ability: Here, only 0-50% produced TNF-α (Figure 45A, left lower panel). Interestingly, overall TNF-α production also decreased from 50% before onset of LN to 39% after onset of LN (Figure 45A, right panel). Additionally, the analysis showed that co-production of TNF-α and IL-2 changed during the disease. In young mice before onset of LN, almost all IL-2^{+} cells (IL-2^{high} and IL-2^{low}) co-produced TNF-α (up to 100%). In contrast, after onset of LN, only IL-2^{high} cells co-produced up to 100% TNF-α.

In summary, TNF-α seemed to be linked with co-production of IL-2 and showed less correlation with IFN-γ production in memory CD4 T cells of NZBxW lupus-prone mice.

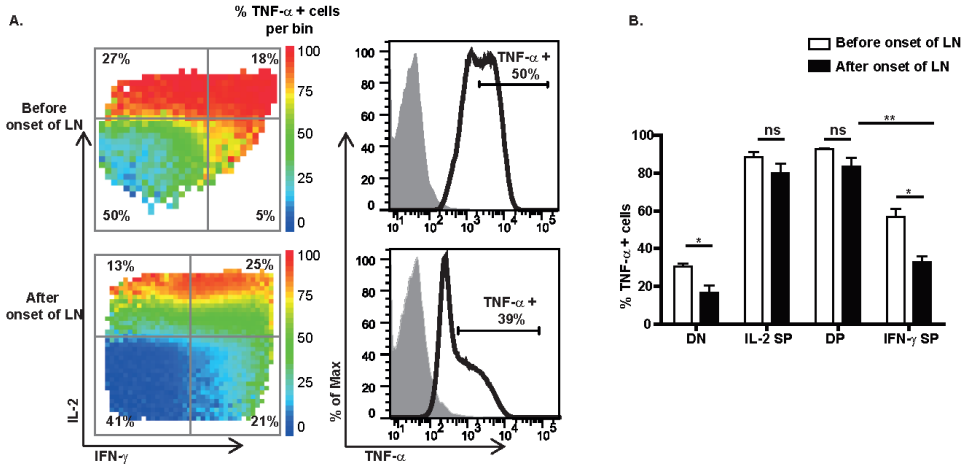

Figure 45. Decreased TNF-α production in IFN-γ SP cells after onset of LN.
(A) Three-parameter plots (left panel) show co-expression of TNF-α, IFN-γ and IL-2 in PMA and Ionomycin stimulated memory CD4 T cells, isolated from spleen and lymph nodes of NZBxW lupus-prone mice before (upper panel) and after onset of lupus nephritis (LN) (lower panel). Cells were graded into bins on the basis of fluorescence intensities of IFN-γ (x axis) and IL-2 production (y axis). Each bin was set up to contain at least 20 cells. In the third dimension, frequencies of TNF-α positive cells per bin are presented as a color-coded heat map. Data were asin h transformed using R software. In the middle row, representative histograms of overall TNF-α production are presented. (B) The bar diagram shows the frequencies of TNF-α producers among DN, IL-2 SP, DP and IFN-γ SP cell subsets of memory CD4 T cells. N=3.

3.7.2. Increased IL-10 expression by IFN-γ SP cells after onset of LN

In analogy to TNF-α, cytokine combinatorial analysis was used to investigate co-expression of IL-10, IL-2 and IFN-γ by memory CD4 T cells of NZBxW mice before and after onset of LN. Flow cytometric analysis of intracellular cytokine staining showed that overall production of IL-10 was about 20% higher in NZBxW lupus-prone mice after onset of LN (24% IL-10$^+$ cells) than in mice before onset of LN (4% IL-10$^+$ cells) (Figure 46A, right panel).

Interestingly, three-parameter visualization revealed that IL-10 production was primarily observed for the subset of IFN-γ SP cells (up to 70%) (Figure 46A, left lower panel); whereas only a minority of DN, IL-2 SP and DP cells appeared to produce IL-10 (0-20%). Moreover, before onset of LN, all bins in all cell subsets appeared dark blue (0% IL-10 producers). In contrast, IL-10 production was generally infrequent before onset of LN (<10%). The few bins showing up to 20% of IL-10 producers within the IFN-γ SP cell subset may be of less importance due to very low overall IL-10 production in young mice before onset of LN (4%).

Taken together, the alterations in TNF-α and IL-10 production seen for IFN-γ SP cell subset seem to be associated with the disease activity.

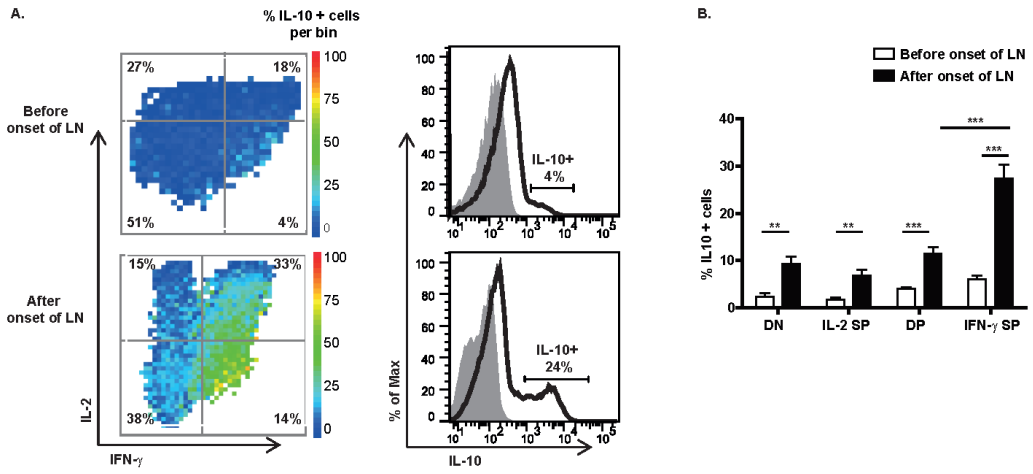

Figure 46. Increased IL-10 production by IFN-γ SP cells after onset of LN.
(A) Three-parameter plots (left panel) show co-expression of IL-10, IFN-γ and IL-2 in PMA and Ionomycin stimulated memory CD4 T cells, isolated from spleen and lymph nodes of NZBxW lupus-prone mice before (upper panel) and after onset of lupus nephritis (LN) (lower panel). Cells were graded into bins on the basis of fluorescence intensities of IFN-γ (x axis) and IL-2 production (y axis). Each bin was set up to contain at least 20 cells. In the third dimension, frequencies of IL-10 positive cells per bin are presented as a color-coded heat map. Data were asin h transformed using R software. In the middle row, representative histograms of overall IL-10 production are presented. (B) The bar diagram shows the

frequencies of IL-10 producers among DN, IL-2 SP, DP and IFN-γ SP cell subsets of memory CD4 T cells. N=3.

In summary, the results of this work revealed by directed investigation of IL-2 and IFN-γ cytokine production by memory CD4 T cells of the NZBxW mouse model of SLE a distinct subpopulation of cells expressing only IFN-γ, but no IL-2, which was increased in manifest disease and may be the main source of elevated IFN-γ levels, as is characteristic for this animal model of SLE. Global gene expression analysis of memory CD4 T cell subsets of diseased NZBxW lupus-prone mice demonstrated certain similarity of IFN-γ SP cells to DP cells, which express both cytokines: IL-2 and IFN-γ. However, at the same time directed expression analysis of selected genes showed that IFN-γ SP cells differed from DP cells in certain aspects. In detail, IFN-γ SP cells were characterized by increased expression of inflammatory chemokine (CXCR6, CXCR4, CCR8, CCR5, CCR2, CCR9) and cytokine (IL12RB2, IFNGR1, IL21R, IL18R1) receptors, increased expression of co-inhibitory (LAG-3, CTLA-4, CD80) and co-stimulatory (CD27, CD40L) receptors, as well as decreased expression of effector cytokines (IL-2, TNF-α, IFN-γ, IL-17), except for IL-10, and a specific pattern of apoptosis-related genes (increased expression of granzymes, but decreased expression of DAPL-1) and transcription factors (increased expression of STAT1, SATB1, IRF1, IRF9, SMAD2, but decreased expression of GATA3 and Myc). All these features might point to a chronically activated phenotype of IFN-γ SP cells of diseased NZBxW lupus-prone mice. In addition, functional analyses revealed that IFN-γ SP cells were tended to decreased proliferation and increased apoptosis, suggesting their reduced viability and functionality.

4. Discussion

4.1. Altered IFN-γ and IL-2 production by memory CD4 T cells in the pathogenesis of SLE

Immunological memory plays a key role in the pathogenesis of many autoimmune diseases, also in systemic lupus erythematosus (SLE), since autoreactive immunological memory can drive chronic inflammation by continuous production of autoantibodies. This was shown by transplantation of memory plasma cells from lupus-prone mice into healthy mice, where they initiated inflammation (Cheng et al., 2013) and by treatment of SLE patients and mice with bortezomib, a proteasome inhibitor, which targets short- and long-lived (or memory) plasma cells, resulting in reduced production of autoantibodies and serum complement levels (Alexander et al., 2018). Further evidence was provided by depletion experiments of the whole immunological memory by immune ablation in patients with various autoimmune manifestations (Alexander et al., 2009), resulting in the elimination of autoreactive immunological memory and the rebuild of the immune system with naive B and T cells (Alexander et al., 2016).

In the late nineties, it was shown that antibody-secreting plasma cells can survive for a long time in special survival niches in the bone marrow and their lifespan is as long as that of memory B cells (Manz et al., 1997; Radbruch et al., 2006; Slifka et al., 1998). Recently, it was shown that plasma cells can survive for about 10 years not only in the bone marrow (Hammarlund et al., 2017), but also in the human gut (Landsverk et al., 2017), contributing to the maintenance of immunological memory und mucosal microbiota (Jahnsen et al., 2018). During chronic inflammatory processes, however, their face changes: Memory plasma cells do not provide immune protection any more, but rather maintain inflammation by continuous secretion of autoantibodies (Cheng et al., 2013; Hiepe et al., 2011; Hoyer et al., 2004; Taddeo et al., 2015).

Memory plasma cells are generated from B cells in germinal centers (Papa and Vinuesa, 2018). This process is strictly dependent on T cell help, provided by specialized follicular helper T cells (T_{FH}) (Grammer et al., 2003; Papa et al., 2017). Besides T_{FH} cells, both T_H1 and T_H2 cells are able to form immune synapses with B cells (Thauland et al., 2008), that are based on antigen-specific interactions (Gardell and Parker, 2017). Since T cells provide help to B cells for antibody

responses (Crotty, 2015), dysregulation of many T cell subtypes (such as T_H1, T_H2, T_{FH}, T_H17 and T_{REG}) has been shown to contribute to the pathogenesis of SLE (Katsuyama et al., 2018; Sawaf et al., 2016).

Indeed, numerous studies demonstrated that SLE is characterized by alterations in T cell phenotype and functions (Katsuyama et al., 2018), such as expansion of the T_H17 population, changes in the T cell receptor repertoire (aggregation of lipid rafts, replacement of CD3ζ chain by the homologous Fc-γR chain) and increased downstream signaling, resulting in continuous activation (Crispín et al., 2008; Crispín and Tsokos, 2010; Moulton and Tsokos, 2011). Additionally, diminished numbers and diminished suppressive capacity of regulatory T cells account for an imbalance between effector and regulatory T cell populations, leading to failure of immune homeostasis and tolerance (Sawla et al., 2012; Suárez-Fueyo et al., 2016).

In this work, it was shown that the frequencies of CD4 T cells, isolated from spleens and lymph nodes of diseased NZBxW lupus-prone mice were decreased and the frequencies of memory CD4 T cells ($CD4^+CD44^+CD62L^-$) were increased, compared to healthy BALB/c mice and NZBxW lupus-prone mice before onset of the disease. This observation is in line with Wang et al., who showed that decreased numbers of CD4 T cells in SLE patients were associated with abnormally high apoptosis, suggesting their diminished functionality (Wang et al., 2005). However, the precise role of memory CD4 T cells is not clear yet.

In this work, drastically increased frequencies of memory CD4 T cells were observed not only in lymphoid tissues, but also blood and visceral organs of diseased NZBxW lupus-prone mice, such as kidneys, lungs and liver, when compared to mice before clinical onset of the disease. In particular, memory CD4 T cells of diseased NZBxW lupus-prone mice were characterized by increased IFN-γ and decreased IL-2 production. The results are in accordance with the findings of Schmidt et al., who found increased infiltration of kidneys with IFN-γ producing CD4 T cells in MRL/lpr and NZBxW lupus-prone mice and their correlation with disease activity (Schmidt et al., 2015).

Aberrant cytokine production in SLE has been a subject of many studies so far. Decreased IL-2 production by T cells and its correlation with SLE activity was shown by several early studies, that suggest a protective role of IL-2 in SLE (Alcocer-Varela and Alarcón-Segovia, 1982; Altman et al., 1981; Linker-Israeli et al., 1983). The hypothesis of a protective role of IL-2 for SLE was

strengthened by the success of treatment of SLE patients and lupus mouse models with low-dose IL-2, which resulted in the reestablishment of T_{REG} homeostasis (Humrich et al., 2010; von Spee-Mayer et al., 2016) and reduced numbers of T_H17 and T_{FH} cells (He et al., 2016). A benefit of low-dose IL-2 immunotherapy has been reported also in other diseases, such as graft-versus-host disease (Koreth et al., 2011; Matsuoka et al., 2013), type 1 diabetes (Hartemann et al., 2013) and hepatitis C virus-induced vasculitis (Saadoun et al., 2011). Besides impaired production of IL-2, impaired response of CD4 T cells to exogenous IL-2 may be a further characteristic of SLE, that is under current discussion (Comte et al., 2017; Comte et al., 2016). In this work, IL-2 production by memory CD4 T cells was found to be decreased not only during active disease, but already prior to clinical onset of the disease in young NZBxW lupus-prone mice, suggesting that an initial imbalance in IL-2 production by memory CD4 T cells may contribute to the disease manifestation.

Next to IL-2, IFN-γ plays an important role in the pathogenesis of SLE (Gottschalk et al., 2015). In this work, IFN-γ production by CD4 T cells and, in particular, memory CD4 T cells was found to be increased in diseased NZBxW lupus-prone mice, which is in line with a previous study by Enghard et al. They found that increased IFN-γ expression by T cells of NZBxW lupus-prone mice correlated with the development of the disease (Enghard et al., 2006). Accordingly, treatment of these mice with IFN-γ led to rapid kidney injury, whereas treatment with anti-IFN-γ antibodies diminished the severity of SLE (Jacob et al., 1987; Schmidt et al., 2015). The role of IFN-γ for driving kidney injury in SLE was strengthened by experiments in IFN-γR deficient MRL/lpr lupus mice, where impaired IFN-γ signaling resulted in reduced deposition of immune complexes and complement component 3 (C3) in the glomerular capillaries of these animals (Haas et al., 1997; Schwarting et al., 1998b). Interestingly, gene expression analysis of this work revealed significantly increased expression of IFN-γR in IFN-γ single producing (IFN-γ SP) subset of memory CD4 T cells of diseased NZBxW lupus-prone mice, when compared to IFN-γ and IL-2 double producers (DP).

Until now, cytokine alterations in SLE were mainly analyzed either by measuring cytokine serum levels or by analyzing cytokine expression in whole peripheral blood mononuclear cells (PBMCs), which include lymphocytes, monocytes, natural killer cells (NK cells) and dendritic cells (Kleiveland, 2015). However, the measurement of total cytokine production cannot distinguish between cell types, which are involved in its altered production. More recent

studies found altered production of several cytokines (IL-2, IFN-γ and IL-10) by isolated CD4 T cells of NZBxW lupus-prone mice, which might be of importance for the pathogenesis of SLE, since their altered expression positively correlated with clinical and serological signs of the disease (Enghard et al., 2006; Humrich et al., 2010). As mentioned, these studies analyzed cytokine expression in whole CD4 T cell population, which included naive, memory and regulatory T cells. To my knowledge, this work is the first, which analyzed altered IL-2 and IFN-γ production in SLE mice with regard to defined memory CD4 T cell subsets. This goal was set to find out phenotypic and functional changes of memory CD4 T cells of diseased NZBxW lupus-prone mice, dependent on their ability to produce IFN-γ and/or IL-2, in order to better understand imbalanced IL-2 and IFN-γ expression by these cells in SLE.

To investigate alterations of defined memory CD4 T cells of diseased NZBxW lupus-prone mice, memory CD4 T cells were purely sorted from whole PMBCs in order to avoid background signals from other cell types. To identify special features of subsets of memory CD4 T cells of diseased NZBxW lupus-prone mice, the cells were further separated into subpopulations, according to their ability of IFN-γ and IL-2 production into IFN-γ^{-}IL-2^{-} double negative (DN), IFN-γ^{-}IL-2^{+} single positive (IL-2 SP), IFN-γ^{+}IL-2^{+} double positive (DP), IFN-γ^{+}IL-2^{-} single positive (IFN-γ SP) cells. Subsequent gene expression analysis revealed distinct gene expression patterns within these subsets. In detail, DP cells were able to co-express multiple cytokines, whereas IFN-γ SP cells lost the ability to express IL-2 as well as other cytokines, among them TNF-α. In fact, the functionality of memory T cells depends on their cytokine production, which was shown following vaccination or viral infection (Wu et al., 2002). Thus, Darrah et al. showed that after vaccination of mice against Leishmania major, CD4 T cells could provide maximal protection, if they were able to co-express multiple cytokines simultaneously, namely IL-2, IFN-γ and TNF-α (Darrah et al., 2007).

The efficiency (or quality) of a T cell response is characterized not only by magnitude (i.e. adequate size of activated memory T cells), but also functionality (i.e. the ability of memory T cells to co-express multiple cytokines) (Lindenstrom et al., 2009). Several models of the development of memory T cells were suggested, which implicate the ability of cells to produce cytokines as one significant feature. Seder et al. suggested a linear model of CD4 T cell differentiation, that highlights the importance of the three cytokines, IL-2, IFN-γ and TNF-α. According to Seder et al., after antigen exposure, CD4 T cells reach their maximal effector

capacity, when producing IL-2, IFN-γ and TNF-α simultaneously. In this stage, they considered memory T cells as “fully functional” cells. Continuous exposure to an antigen, however, may lead to progressive loss of cytokine production and thus their effector functions and memory potential. In this stage, the cells produce only IFN-γ and are short-lived. The latter they referred to “terminally differentiated effector cells” (Figure 47) (Seder et al., 2008).

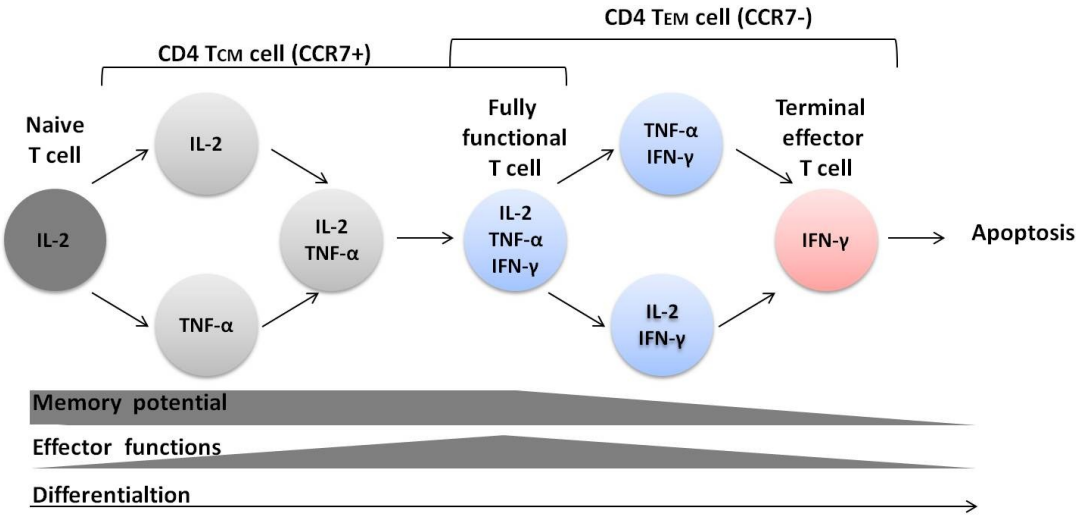

Figure 47. Linear model of T_H1 cell differentiation and memory development.
After antigen exposure, naive T cells get activated and differentiate into effector cells, undergoing distinct phases of cytokine production and finally apoptosis. The cells reach their most potent status of effector functions and memory capacity, when producing IL-2, IFN-γ and TNF-α simultaneously. Continuous exposure to the antigen leads to progressive loss of cytokine production and effector functions of the cell (from (Seder et al., 2008)).

Besides this linear model of memory T cell differentiation, other models were suggested, as well. Thus, Kaech et al. presented their “decreasing-potential hypothesis” of memory T cell differentiation, which suggests that upon continuous antigen stimulation, for example during chronic viral infection, effector functions of T cells decrease and the cells undergo apoptosis faster, resulting in reduced functionality and reduced numbers of memory T cells, a status referred to “dysfunctional effector T cells” (Kaech et al., 2002).

A third model of T cell differentiation under conditions of antigen persistence, for example during chronic viral infections and tumors, introduces the term “exhausted T cells” (Wherry, 2011; Zajac et al., 1998). According to Werry et al., exhausted T cells are characterized by a step-wise loss of their ability to produce cytokines: First, they lose IL-2, then TNF-α, whereas IFN-γ production seems most stable and is lost only with progression of exhaustion. Further,

exhausted T cells are characterized by reduced proliferative capacity and increased expression of co-inhibitory molecules (Kahan et al., 2015)

According to the above presented models of T cell differentiation (Kaech et al., 2002; Seder et al., 2008; Wherry, 2011), IFN-γ SP cells of diseased NZBxW lupus-prone mice may correspond to "terminally differentiated effector T cells"/ "dysfunctional effector T cells"/ or "exhausted T cells". At the same time, it was found in this work that IFN-γ SP cells of diseased NZBxW lupus-prone mice displayed increased expression of co-inhibitory receptors, such as CTLA-4 and LAG-3, increased expression of co-stimulatory receptors, such as CD27 and CD40L, and increased expression of many inflammatory chemokine receptors, such as CXCR6, CXCR4, CCR8, CCR5, CCR2, CCR9, which might rather indicate a "hyperactivated" phenotype of IFN-γ SP cells.

DP cells of diseased NZBxW lupus-prone mice in their turn may represent a cell subset with the most potent effector functions ("fully functional T cells"), since DP cells were characterized by co-production of IL-2, IFN-γ and TNF-α. Thus, one may speculate that the controversy on the role of IFN-γ in SLE could be explained by the fact that IFN-γ is produced by two subpopulations of memory CD4 T cells: Fully functional cells (i.e. DP) and cells with impaired production of immunomodulatory cytokines (IL-2 and TNF-α) as well as increased expression of co-inhibitory molecules and inflammatory chemokines (i.e. IFN-γ SP).

However, the explicit roles of distinct subpopulations of memory CD4 T cells (DN, IL-2 SP, DP and IFN-γ SP cells) in SLE and whether they participate in the induction of autoimmunity remains to be determined. Adoptive-transfer experiments may be helpful to answer these questions. Furthermore, experiments based on selective targeting of these cell subsets may shed light on the mechanisms of how distinct cell types might contribute to the disease development.

4.2. Multi-step cell purification enabled informative gene expression analysis

Pathological immunological memory is generated under conditions of continuous antigen exposure. By continuous antigen exposure pathogenic memory T cells "get adapted" to the conditions of chronic inflammation and start to express new genes. For instance, *Twist-related protein 1 (Twist1)* and *Homebox only protein (Hopx)* have been described to be specifically expressed in pathogenic memory T_H1 cells. On the one hand, *Twist1* inhibits expression of pro-

inflammatory genes and on the other hand, it contributes to the longevity of pathological memory T cells by the inhibition of pro-apoptotic genes. Thus, expression of *Twist1* enables pathological memory T cells to drive chronic inflammation (Niesner et al., 2008). The *Hopx* gene was identified as a selective regulator of the survival of T_H1 memory cells. Its expression is induced by T-bet, the master transcription factor of T_H1 cells, and increases upon repetitive stimulation of the T cell receptor, as found under conditions of continuous antigen exposure. By contrast, in naive cells expression of *Hopx* is low. Remarkably, *Hopx* deficient T_H1 cells cannot induce chronic inflammation in experimental murine models of transfer-induced colitis and arthritis, demonstrating the important role of *Hopx* in autoimmunity (Albrecht et al., 2010).

Therefore, the investigation of alterations within the gene expression program during autoimmunity has been proven a powerful approach to identify autoimmunity-driving factors and to shed light onto the processes of pathological immunological memory for chronic inflammation. With that motivation, gene expression analyses were performed in this work in order to reveal gene expression profiles of memory CD4 T cell subsets that may be associated with chronic inflammation in SLE.

In recent years, gene expression profiling has been intensively used as a tool for identification of diagnostic and prognostic markers, especially in chronic inflammatory diseases. Though several gene expression datasets from SLE patients have been already published, most of them originated from unsorted PBMCs. Because of this mixture of a variety of cell types, these microarray datasets had high background information and identified only very prominent dysregulated pathways. Thus, the only common expression pattern observed across all SLE cell subsets was so called type I interferon (IFN-α) signature (genetic association with IFN-α-related pathways) (Bae and Lee, 2018; Frangou et al., 2013). However, by this approach even the impact of type II interferon (IFN-γ) could not be detected. By contrast, in another study the microarray analysis of sorted CD4 and CD8 T cells, B cells, monocytes and neutrophils from SLE patients showed great advantage over unsorted PBMCs for the indication of the pathological relevance of different cell subsets (Lyons et al., 2010). In this study, 1607 differentially expressed genes were identified between SLE and control groups over all five cell types, however, 86% of these genes was not seen in PBMCs. Hence, gene expression analyses of sorted cell subsets were able to reveal additional essential pathways in SLE, i.e. plasmablast (immunoglobulin) and granulopoesis (proteins expressed in immature granulocytes) signatures

(Lyons et al., 2010). These features could be used as differential criteria between SLE and ANCA-associated small vessel vasculitis (AAV), as the IFN-α and plasmablast signatures were not seen in AAV, whereas the granulopoesis signature was seen in both diseases (Lyons et al., 2010). Recently, analysis of available microarray datasets of CD4 T cells revealed shared gene expression profiles and biological mechanisms between patients with pemphigus and SLE, suggesting novel potential targets for therapy in these diseases (Sezin et al., 2017). The impact of cell sorting on the quality of microarrays was also shown by another study, where even hidden gene expression profiles of small cell subsets could be discovered by accurate cell sorting (Szaniszlo et al., 2004).

In current work, a multi-step method of cell purification was employed. First, CD4 T cells were isolated from PBMCs using magnetic-activated cell sorting (MACS). Second, memory CD4 T cells ($CD4^+D44^+CD67L^-$) were isolated out of the whole population of CD4 T cells using fluorescent-activated cell sorting (FACS). Third, using a cytokine secretion assay memory CD4 T cells were separated, according to their ability to express IL-2 and/or IFN-γ after stimulation for 5 h with PMA and Ionomycin. This proceeding allowed for isolation of four subpopulations of living memory CD4 T cells by FACS, namely (1) IFN-γ^-IL-2^- double negative (DN); (2) IFN-γ^-IL-2^+ single positive (IL-2 SP); (3) IFN-γ^+IL-2^+ double positive (DP) and (4) IFN-γ^+IL-2^- single positive (IFN-γ SP) cells, and finally, subset-specific gene expression analyses as well as further culture *in vitro*.

Taken together, multi-step cell purification is a good approach for defining small cell subsets. In addition, gene expression analysis of these defined cell subsets is more informative in comparison with that of PBMCs, since the undesired background information from other cells in repertoire is significantly reduced. This approach enabled to show that the subpopulations of memory CD4 T cells of diseased NZBxW lupus-prone mice were defined by unique gene expression profiles. The disadvantage of the multi-step cell purification is the loss of large amounts of cells during each step of cell sorting, which in its turn increases the need in mice.

4.3. Altered gene and protein expression of IFN-γ SP cells of diseased NZBxW lupus-prone mice

As mentioned above, gene expression analysis of purified subpopulations of memory CD4 T cells of diseased NZBxW lupus-prone mice revealed that these cell subsets were defined by unique gene expression profiles. In more detail, IFN-γ SP cells were characterized by a different phenotype than DP cells of diseased NZBxW lupus-prone mice. Among differentially expressed genes between IFN-γ SP vs. DP cells of diseased NZBxW lupus-prone mice, several functional groups can be distinguished, part of which were also examined on protein level. In detail, IFN-γ SP cells displayed:

(1) increased expression of inflammatory chemokine receptors (s. 4.3.1.),
(2) increased expression of co-inhibitory receptors (s. 4.3.2.),
(3) increased expression of IFN-regulated transcription factors (s. 4.3.3.),
(4) decreased expression of effector cytokines, except IL-10 (s. 4.3.4.),
(5) increased expression of apoptotic markers, except DAPL-1 (s. 4.3.5.)

In addition to the impact of gene expression profiling for the pathogenesis of SLE (s. 4.2.), valuable information about the role of a range of genes in SLE has been provided by gene deletion experiments in lupus mouse models by recent studies. Among these disease-effecting species, several were found to be differentially expressed between IFN-γ SP vs. DP cells of diseased NZBxW lupus-prone mice in the current dataset (Table 12). In the following sections, these and further potentially meaningful candidates from the set of differentially expressed genes between IFN-γ SP vs. DP cells will be discussed in the context of SLE.

Table 12. Effects of gene manipulation on SLE manifestation

Gene manipulation	Mouse strain	Effects on SLE	Reference	Expression in IFN-γ SP cells (own data)
***Stat1*$^{-/-}$** ***Irf9*$^{-/-}$**	Pristane-induced mouse model of SLE	↓auto-ABs	(Thibault et al., 2008)	↑*Stat1* ↑*Irf9*
***Ifr1*$^{-/-}$**	MRL/lpr lupus mice	↓dermatitis, ↓anti-dsDNA ABs, ↓proteinuria, ↓GN, ↑T_{REG} cells	(Reilly et al., 2006)	↑*Irf1*
***Il10*$^{-/-}$**	MRL/lpr lupus mice	↑skin lesions, ↑lymphadenopathy,	(Yin et al., 2002)	↑*Il10*

		↑GN, ↑mortality ↑IFN-γ production		
***Tnfr1*$^{-/-}$** ***Tnfr2*$^{-/-}$**	C57BL/6 mice backcrossed into SLE-prone NZM 2328 mice.	↑GN, ↑anti-dsDNA ABs, ↑T_{EM} cells, T_H17 gene profile	(Jacob et al., 2009)	Only ↑*Tnfrsf1b (Tnfr2)*
***Tnf*$^{-/-}$**	NZB hemizygous mice	↑GN, ↑ANA, ↑B cell proliferation	(Kontoyiannis and Kollias, 2000)	↓*Tnf*
***Il17a*$^{-/-}$**	MRL/lpr lupus mice	No effect on LN	(Schmidt et al., 2015)	↓*Il17a*
***Il21r*$^{-/-}$**	SLE-prone BXSB-Yaa mice	↓GN, ↓mortality	(Bubier et al., 2009)	↑*Il21r*
***Infgr*$^{-/-}$**	MRL/lpr and NZBxW lupus-prone mice	↓ GN, ↓anti-dsDNA ABs, ↓anti-histone ABs	(Haas et al., 1998) (Schwarting et al., 1998b)	↑*Irfgr1*
***B7.1*$^{-/-}$ (CD80)**	MRL-lpr lupus mice	↑GN	(Liang et al., 1999)	↑*Cd80*

GN – glomerulonephritis, AB – antibody, ANA – anti-nuclear autoantibody, LN – lupus nephritis

4.3.1. Increased expression of inflammatory chemokines by IFN-γ SP cells

Upon activation, naive T cells differentiate into functionally mature memory and effector T cells. This differentiation program brings changes not only in their cytokine repertoire, but also in the expression pattern of chemokine receptors, which guide the migratory behavior of the cells (Campbell et al., 1999; Kim et al., 1998). In this work, gene expression analysis revealed that IFN-γ SP cells of diseased NZBxW lupus-prone mice expressed significantly increased levels of several inflammatory chemokines, when compared to DP cells, among those CCR2 and CCRL2 as well as CCR5, CXCR4 and CXCR6. Accordingly, potential pathogenic roles of these chemokines were shown for SLE in various studies and will be discussed in the following section.

It was observed in this work that CCR2 and its ligand CCRL2 were increased in IFN-γ SP cells of diseased NZBxW lupus-prone mice. The role of the chemokine CCR2 in SLE was shown in previous studies, demonstrating that CCL2 was responsible for the attraction of immune cells to inflamed kidneys during lupus nephritis (Noris et al., 1995; Wada et al., 1996). In accordance with these findings, Enghard et al. showed that the urine samples of SLE patients were enriched

with CD4 T cells, expressing high levels of CCL2 and CXCL10, which positively correlated with the activity of lupus nephritis (Enghard and Riemekasten, 2009). Interestingly, Duarte-Garcia et al. showed that increased levels of CCL2 in spinal fluid of SLE patients were associated with mental dysfunctions (Duarte-Garcia et al., 2018). Moreover, the association of increased CCL2 levels with cognitive impairment was shown not only during SLE, but also during dementia (Kimura et al., 2018; Westin et al., 2012).

Besides CCR2 and CCL2, the expression of CCR5 was increased in IFN-γ SP cells of diseased NZBxW lupus-prone mice, as shown in this work. The functional role of CCR5 in SLE has been investigated in both animal models and humans with SLE so far. Remarkably, in MRL/lpr lupus mice and in humans with SLE renal and urinary mRNA expression of CCR5, respectively, was increased, correlating with disease activity (Chan et al., 2006; Perez de Lema et al., 2001). Moreover, gene transfer of CCR5 into kidneys of MRL/lpr lupus mice resulted in exacerbation of inflammation (Ishikawa et al., 2001). Al-Saleh et al. showed that CCR5 expression was increased on CD4 T cells of patients with active SLE (Al-Saleh and el-Eissawy, 2006). Furthermore, CCR5 haplotypes were associated with a higher risk for SLE and kidney injury (Mamtani et al., 2008). In contrast, mice lacking CCR5 developed reduced symptoms of experimental autoimmune encephalomyelitis (EAE) (Gu et al., 2016); and increased levels of CCR5 were declining under methotrexate therapy in patients with rheumatoid arthritis (Boiardi et al., 1999).

Further in this work, the expression of CXCR4 was found to be increased in IFN-γ SP cells of diseased NZBxW lupus-prone mice, when compared to DP cells. A role of CXCR4 in SLE was shown by Ma et al. by investigating $TLR4^{+}CXCR4^{+}$ plasma cells, which were increased in blood and kidneys of SLE patients and mice and were associated with kidney injury (Ma et al., 2018). Furthermore, CXCR4 expression was shown to be increased on B cells and correlated with disease activity and kidney injury in patients with SLE (Zhao et al., 2017). However, the impact of CXCR4 on T cell activity in SLE has not been investigated, yet.

In addition, the expression of CXCR6 was increased in IFN-γ SP cells of diseased NZBxW lupus-prone mice, as shown in this work. The role of CXCR6 for inflammation is currently under discussion (Hu et al., 2016). CXCR6 was shown to be predominantly expressed on memory T cells, whereby $CXCR6^{-}$ and $CXCR6^{+}$ memory T cells differed from each other in their cytokine production pattern. In detail, $CXCR6^{+}$ memory CD4 T cells were shown to produce higher frequencies of IFN-γ and moderate frequencies of TNF-α and IL-2, which is in accordance with

the observations in this work. Moreover, CXCR6^{+} memory T cells were highly enriched in inflamed tissues, indicating their role in organ damage in SLE (Kim et al., 2001). Correspondingly, memory T cells, associated with the CXCL16-CXCR6 pathway were shown to be potentially pathogenic in several inflammatory conditions, such as rheumatoid arthritis, Crohn's disease, autoimmune colitis and inflamed liver (Diegelmann et al., 2010; Ruth et al., 2006; Sato et al., 2005; Tomita et al., 2008; Uza et al., 2011; van der Voort et al., 2005).

As discussed here, chemokine receptors are significantly involved in SLE and other autoimmune diseases. Their increased expression on IFN-γ SP cells of diseased NZBxW lupus-prone mice point to the altered phenotype of IFN-γ SP cells. It is of high interest to investigate, whether increased expression of certain pro-inflammatory chemokine receptors might be a relevant feature of IFN-γ SP cells for the pathogenesis of SLE.

4.3.2. Increased expression of co-inhibitory receptors by IFN-γ SP cells

Co-inhibitory receptors are components of lipid rafts on the cell surface. They are negative regulators of the T cell receptor activation (Walunas et al., 1996; Walunas et al., 1994). Accordingly, enhanced expression of co-inhibitory receptors leads to inhibition of cell cycle processes, inhibition of effector functions (such as suppression of the production of effector cytokines, e.g. IL-2), exhaustion and apoptosis (Chen and Flies, 2013).

In this work, expression of two important co-inhibitory receptors, CTLA-4 and LAG-3, was shown to be increased in IFN-γ SP cells of diseased NZBxW lupus-prone mice, when compared to DP cells. Co-inhibitory receptors, such as PD-1, LAG-3, CTLA-4, TIM-3 were shown to be involved in many autoimmune diseases (Kasagi et al., 2011; Qu et al., 2009; Song et al., 2011; Wang et al., 2014), cancers (Andrews et al., 2017; Daud et al., 2016) and chronic viral infections (Pauken and Wherry, 2015). Though a significant role of LAG-3 has been shown in autoimmunity, viral infections and cancers, it is still unclear, how LAG-3 participates in these processes (Anderson et al., 2016). Even less is known about the role of LAG-3 in SLE. The results of this work showed that IFN-γ SP cells of diseased NZBxW lupus-prone mice had higher expression of LAG-3. The latter was shown to inhibit effector functions of T cells, among them production of effector cytokines (Hannier et al., 1998). Therefore, one can hypothesize that the loss of IL-2 production by IFN-γ SP cells might be associated with the increased expression of

LAG-3 by these cells. Indeed, IL-2 SP cells expressed (in contrast to IFN-γ SP cells) very few (almost irrelevant) amounts of LAG-3.

Next to LAG-3, expression of a second co-inhibitory receptor, CTLA-4, was increased in IFN-γ SP cells. Several studies investigated the role of CTLA-4 in SLE. In detail, the expression of CTLA-4 was shown to be increased in T cells from patients with active SLE (Hirashima et al., 2004; Jury et al., 2010; Liu et al., 1998). In addition, in MRL/lpr lupus mice increased expression of CTLA-4 isoforms 1/4 was associated with disease exacerbation, activation of T and B cells and high expression levels of IFN-γ (Ichinose et al., 2013). Furthermore, blockade of the CTLA-4 pathway in lupus-prone mice led to the amelioration of the disease (Daikh et al., 2006; Finck et al., 1994b).

Taken together, increased expression of co-inhibitory receptors together with increased expression of inflammatory chemokine receptors by IFN-γ SP cells point to an altered phenotype of these cells. However, further investigations are necessary to elucidate, if IFN-γ SP cells contribute to the pathogenesis of SLE upon these features.

4.3.3. Increased expression of IFN-regulated transcription factors by IFN-γ SP cells

IFN-γ signaling is mediated by binding to its specific receptor (IFN-γR), which in turn induces the activation of several kinases and the translocation of Signal transducer and activator of transcription 1 (STAT1) into the nucleus. The transcription factor STAT1 binds to the promoters and the regulatory regions of IFN-γ-regulated genes and induces gene expression (Ramana et al., 2002). Among the STAT1 targets of the IFN-γ signaling pathway are SMAD family members, interferon regulatory factors (IRF) and proteins of cell cycle regulation (e.g. Myc) (Ramana et al., 2000; Saito et al., 2000). STAT1 and all known members of the IRF family (IRF1-9) are required for the induction of IFN-regulated genes and interferons themselves (Durbin et al., 1996; Kimura et al., 1996; Taniguchi et al., 2001). Remarkably, several of these genes (*Stat1, Smad2, Irf1, Irf9*) were upregulated and *Myc* was downregulated selectively in IFN-γ SP cells of diseased NZBxW lupus-prone mice.

Thilbaut et al. investigated the potential pathogenic role of IFN-regulated transcription factors in SLE by directed gene deletion (Table 13) (Thibault et al., 2008). Remarkably, deletion of *Stat1*

and *Irf*s (*Irf1* and *Irf9*) resulted in reduced autoantibody production and amelioration of the disease, pinpointing them as important factors in isotype switching process for pathogenic autoantibody production in SLE mice (Reilly et al., 2006; Thibault et al., 2008). Furthermore, deletion of *Irf1* in murine mercury-induced systemic autoimmunity, a condition similar to SLE, resulted in amelioration of the disease, as well (Pollard et al., 2012). In monocytes of SLE patients, IRF1 binding was shown to be increased and was associated with increased histone acetylation, suggesting that IRF1 might alter the epigenome during disease development (Zhang et al., 2015).

Dong et al. suggested that also the STAT1 signaling pathway might be important for the pathogenesis of lupus nephritis, as they observed increased expression and activation of STAT1, correlating with kidney inflammation in MRL/lpr lupus mice (Dong et al., 2007). Indeed, STAT1 expression was increased in PBMCs and peripheral blood leucocytes of patients with SLE and correlated with disease activity (Dominguez-Gutierrez et al., 2014; Karonitsch et al., 2009). In contrast, expression levels of miR-145, a suppressor of STAT1, were shown to be decreased in T cells of SLE patients and were associated with increased expression of STAT1 and severity of kidney pathology in these patients (Lu et al., 2013). Recently, it was shown that STAT1 protein levels were increased in CD4 T cells of patients with active SLE (Goropevsek et al., 2017).

These studies highlight the importance of STAT1 transcriptional activity in the pathogenesis of SLE and suggest STAT1 as a therapeutic target. Thus, a *Stat1*$^{-/-}$ knock out in MRL/lpr lupus mice resulted in reduced proteinuria, autoantibody production and glomerulonephritis (GN). However, these mice developed severe tubulointerstitial nephritis (TIN) and showed increased proportion of T_H17 cells (Yiu et al., 2016), demonstrating that despite its pro-inflammatory features, STAT1 is important for the inhibition of T_H17 cells.

Another target of STAT1 activity is *Tbx21*, encoding the master transcription factor of T_H1 cells, T-bet (Christie and Zhu, 2014), which is also essential for IFN-γ production by T_H1 cells (Murray et al., 2002). It is known that over-expression of T-bet leads to increased proteinuria and sever glomerulonephritis in lupus-prone mice. Furthermore, elevated ratios of T_H1:T_H2 cells with respectively increased expression of IFN-γ have been described in SLE (Shimohata et al., 2009). However, T-bet was not differentially expressed between IFN-γ SP and DP cell subsets of diseased NZBxW lupus-prone mice in this work, which might be reasonable, as both subsets express IFN-γ. Also other transcription factors, such as NFAT, NF-κB and AP-1, that are essential

for the expression of IFN-γ (Zimmermann et al., 2016), exhibited equal expression levels between IFN-γ SP and DP cells of diseased NZBxW lupus-prone mice.

4.3.4. Pleiotropic functions of IL-10 and TNF-α in SLE

4.3.4.1. Three-parameter visualization tool for cytokine co-expression analysis

State-of-the-art flow cytometers allow for the analysis of up to 30 parameters simultaneously. Thus, multi-parameter flow cytometry is a powerful technique, which enables simultaneous assessment of phenotype and functions of a single cell and allows for defining distinct cell populations (Cossarizza et al., 2017). In contrast, a single parameter does not reflect full functional potential of the cell (Seder et al., 2008). Therefore, with the advancement of flow cytometry the need to develop useful data analysis tools increases steadily.

The application of a 3D tool of flow cytometry data, which was recently established in the "Signal Transduction" working group at the German Rheumatism Research Centre, allowed for combinatorial analysis of at least three cytokines simultaneously. With the help of this tool, differences in cytokine co-expression could be visualized in mice before and after onset of lupus nephritis investigating the prevalence of IL-10 or TNF-α production in the spectrum of IL-2 and/or IFN-γ producing cells (s. 4.3.4.2. and 4.3.4.3.). The tool proofed to be a valuable method with advantages like a high grade of intuitiveness and reproducibility, and complements the list of other programs for flow cytometry data analysis (such as VISNE and SPADE).

4.3.4.2. Increased expression of IL-10 by IFN-γ SP cells

IL-10 is an important immunomodulatory cytokine which regulates differentiation of many cell types and was shown to be able to inhibit activation of antigen presenting cells (APCs) (de Waal Malefyt et al., 1991) and T_H1 cells (Darrah et al., 2010) and can potentially suppress inflammatory responses (Moore et al., 2001). Remarkably, the *Il10* gene is strongly associated with SLE susceptibility (D'Alfonso et al., 2000). However, the role of IL-10 in SLE appears to be complex.

Gene expression and protein expression analyses in this work revealed increased IL-10 production by IFN-γ SP cells of diseased NZBxW lupus-prone mice, when compared to DP cells;

furthermore, overall expression levels of IL-10 by memory CD4 T cells were significantly higher in diseased mice than in controls. In accordance, several studies showed that the production of IL-10 in SLE patients was increased and its increased levels positively correlated with disease activity. In addition, a small group of SLE patients treated with anti-IL-10 antibodies reported about reduction of disease activity (Csiszar et al., 2000; Hagiwara et al., 1996; Houssiau et al., 1995; Llorente et al., 2000; Llorente et al., 1995; Park et al., 1998). Furthermore, continuous treatment of young NZBxW lupus-prone mice with anti-IL-10 antibodies delayed the onset of the disease (Ishida et al., 1994). Interestingly, Enghard et al. showed that CD4 T cells, isolated from spleens of NZBxW lupus-prone mice had increased expression of IL-10, which correlated with anti-dsDNA titers and proteinuria, but not with the age of these mice (Enghard et al., 2006). According to these data, increased IL-10 expression in SLE might point to an inflammation-driving role in the pathogenesis of SLE. The gain of pathogenic features with disease progression might be explained by a failure of IL-10 producing T cells to suppress T_H1 responses and APC activities (Darrah et al., 2010; de Waal Malefyt et al., 1991; Moore et al., 2001), resulting in increased activation and differentiation of B cells into antigen-producing plasma cells (Beebe et al., 2002), which in their turn are key components of the pathogenesis of SLE (Alexander et al., 2018).

In contrast to these findings, Langer et al. found that IL-10 expression was decreased in T cells, stimulated *in vitro* with $SmD1^{83-119}$, when compared to healthy controls (Langer et al., 2007). $SmD1^{83-119}$ is an immunogenic peptide for autoantibody response in SLE (Riemekasten et al., 1998). In support, another study showed that IL-10 levels were decreased in T cells from SLE patients, although there was no correlation with disease activity. In the same study, however, the patients with high anti-dsDNA antibody titers in the sera had also higher expression levels of IL-10 (Chang et al., 2002). Yin et al. showed that *Il10* gene deletion in MRL/lpr lupus mice resulted in exacerbation of the disease due to enhanced IFN-γ production in both CD4 and CD8 T cells (Yin et al., 2002). Notably, clinical exacerbation of the disease as well as increased autoantibody production due to IL-10 deficiency was seen at early time points (2-2.5 month of age), demonstrating a protective role of IL-10 at an early stage of the disease (Yin et al., 2002).

In this study, combinatorial flow cytometric analysis of IL-10, IL-2 and IFN-γ showed that in diseased NZBxW lupus-prone mice IL-10 was produced predominantly by IFN-γ SP cells, and rather not by DN, IL-2 SP or DP cells. Furthermore, very low IL-10 production was observed by

memory CD4 T cells in young NZBxW lupus-prone mice (1-2.5 month of age) before clinical onset of SLE.

Thus, these findings indicate the complex, but significant role of IL-10 in the pathogenesis of SLE. For now, the role of IL-10 in SLE is still controversial and seems to be dependent on the stage of the disease, revealing protective function at early time points and pathogenic function in late manifest disease.

4.3.4.3. Decreased expression of TNF-α by IFN-γ SP cells

The role of TNF-α in SLE is controversial, as well, revealing protective and pathogenic functions. TNF-α is an important cytokine, which mediates a variety of cellular functions, such as cell differentiation, proliferation and apoptosis (Beutler and Cerami, 1988; Goeddel et al., 1986; Kollias and Kontoyiannis, 2002; Kollias et al., 2002; Sugarman et al., 1985). Accordingly, TNF-α blockers are used for the treatment of rheumatoid arthritis, juvenile arthritis, psoriasis arthritis, plaque psoriasis, ankylosing spondylitis, uncreative colitis and Crohn's disease (data of WebDM and DGRh). Therefore, the understanding of functions and signaling of TNF-α is of particular importance.

In this work, combinatorial flow cytometric analysis of TNF-α, IFN-γ and IL-2 showed that TNF-α expression was significantly decreased in IFN-γ SP cells after onset of nephritis in NZBxW lupus-prone mice, whereas IFN-γ SP cells of young mice (before onset of the disease) still displayed high levels of TNF-α production. Furthermore, the co-production of TNF-α and IL-2 changed during the disease, namely in diseased mice only IL-2^{high} cells were able to co-produce 100% TNF-α, whereas in young mice (before onset of the disease) IL-2^{high} and IL-2^{low} cells were able to co-produce 100% TNF-α. In this context, TNF-α production seemed to be dependent on the ability of the cell to co-produce IL-2 and less on IFN-γ co-production.

A protective role of TNF-α in SLE was shown by an early study with administration of TNF-α to young NZBxW lupus-prone mice, which led to the suppression of renal disease (Gordon et al., 1989). In accordance, TNF-α deficiency in NZB mice (NZB is a mouse strain with limited manifestation and activity of lupus (Perry et al., 2011)) resulted in the acceleration of autoimmunity and nephritis, demonstrating its immunomodulatory functions in this mouse model of SLE (Kontoyiannis and Kollias, 2000).

In SLE patients, the role of TNF-α seems also to be bidirectional. There are many reports that the treatment of patients with rheumatoid arthritis or autoimmune colitis with TNF-α blockers resulted in SLE-like symptoms and autoantibody production (Feldmann et al., 1996; Lin et al., 2008; Sandborn and Hanauer, 1999; Sicotte and Voskuhl, 2001). On the other side, in a small group of patients with SLE the administration of TNF-α blockers was shown to be beneficial (Aringer and Smolen, 2008; Aringer et al., 2007). The distinct roles of TNF-α blockers in two groups of SLE patients might be explained by their genetic predisposition to develop SLE, since not all patients treated with TNF-α blockers develop lupus-like symptoms (Jacob et al., 1990).

Pro-inflammatory and immunomodulatory functions of TNF-α might be explained by the existence of two distinct TNF receptors: TNFR1 and TNFR2. TNFR1 mediates TNF-induced inflammation and cell death, whereas TNFR2 mediates proliferation and programmed cell death of mature T cells (Baud and Karin, 2001; Tartaglia et al., 1993; Tartaglia et al., 1991; Zheng et al., 1995). Gene expression analysis of this work showed that the expression of TNFR2 was increased in IFN-γ SP cells, whereas the expression of TNFR1 was not significantly different between IFN-γ SP and DP cells of diseased NZBxW lupus-prone mice. As TNFR2 mediates rather immunomodulatory functions of TNF-α, the role of TNFR2 seems to be weak, since the expression of TNF-α was significantly decreased in IFN-γ SP cells, which was associated with massive proteinuria and sever glomerulonephritis in these mice.

TNF-α is considered to inhibit T_H17 cell differentiation (Zaba et al., 2007). Remarkably, Jacob et al. reported that double deletion of *Tnfr1* and *Tnfr2*, but not single deletion of either TNF receptor resulted in accelerated lupus nephritis and accumulation of memory T cells with a pathogenic T_H17 gene profile and increased production of IL-17 (Jacob et al., 2009). In IFN-γ SP cells, however, decreased expression levels for the *Il17* gene (both *Il17a* and *Il17f*) were observed. In detail, protein expression was not detectable by intracellular IL-17 staining using flow cytometry (data not shown), which might be explained by enhanced expression of TNFR2 by IFN-γ SP cells.

Though IL-17 is associated with autoimmunity (Lee et al., 2012), *Il17* gene deletion in MRL/lpr lupus mice did not affect the morphologic or functional parameters of lupus nephritis. In addition, treatment of NZBxW lupus prone mice with anti-IL-17 antibody did not affect clinical course of lupus nephritis, suggesting rather a minor role of IL-17 in the pathogenesis of SLE in MRl/lpr and NZBxW lupus-prone mice (Schmidt et al., 2015).

4.3.5. IFN-γ SP cells tend to increased apoptosis

One predominant hallmark of the pathogenesis of SLE is enhanced apoptosis and impaired clearance of apoptotic cells, since debris of apoptotic cells accumulate in the glomerular capillaries and lead to end organ damage (Alexander et al., 2015; Cohen, 2006). Earlier studies showed that the apoptosis of lymphocytes from SLE patients was increased compared to the patients with rheumatoid arthritis and correlated with disease activity (Emlen et al., 1994). Analysis of apoptosis-related genes in this work revealed that the expression of granzymes was increased in IFN-γ SP cells of diseased NZBxW lupus-prone mice, when compared to DP cells. Increased production of granzyme B might point to the pro-inflammatory feature of these cells, since release of granzyme B is known to induce apoptosis of target cells (Lord et al., 2003). In contrast, the expression of another apoptosis-related gene, *Death-associated protein ligand 1 (Dapl1)*, was decreased in IFN-γ SP cells of diseased NZBxW lupus-prone mice, when compared to DP cells. *Dapl1* is a relatively unknown gene, however, it was found to be within the top 10 of differentially expressed genes in current microarray dataset. The action of DAPL-1 has been connected to death-associated protein (DAP), which acts as a positive mediator of programmed cell death upon induction by IFN-γ (Deiss et al., 1995). In addition, DAPL-1 was shown to act as a cell proliferation receptor in retinal pigment epithelial cells (Ma et al., 2017). However, its association with SLE has not been described yet. According to these data, diminished DAPL-1 expression in IFN-γ SP cells of diseased NZBxW lupus-prone mice might point to an increase of cell proliferation. Paradoxically, this is controversial to the following observations in this work, which showed rather an increased apoptosis and decreased proliferation rates of IFN-γ SP cells. Hence, gene enrichment analyses of this work revealed that IFN-γ SP displayed increased expression of genes, associated with regulation of cell death and vice versa, decreased expression of genes, associated with regulation of mytosis, when compared to DP cells. Moreover, subsequent proliferation and apoptosis assays supported the hypothesis that IFN-γ SP cells tended to proliferate less, but tended to have higher apoptotic capacity than DP cells, suggesting their limited functionality and viability. In accordance, other studies showed that the apoptosis of CD4 T cells was increased in SLE patients and the rate of apoptotic T cells correlated with disease activity; furthermore, increased apoptosis of CD4 T cells was shown to be linked to increased IL-10 production (Wang et al., 2005; Yang et al., 2015). In addition, reduced proliferation of CD4 and CD8 cells had been described particularly in inflamed kidneys of MRL/lpr lupus mice (Tilstra et al., 2018).

4.4. IFN-γ SP cells share features of hyperactivated and exhausted cells

Next to increased apoptosis and decreased proliferation potentials of IFN-γ SP cells of diseased NZBxW lupus-prone mice, these cells were further characterized by reduced production of effector cytokines, increased expression of pro-inflammatory chemokines and co-inhibitory and co-stimulatory receptors. In accordance with these data, Tilstra et al. showed that kidney infiltrating T cells of MRL/lpr lupus mice displayed decreased expression of effector cytokines and increased expression of co-inhibitory receptors along with decreased proliferative capacity; moreover, they found that the transcription profile of these cells was similar to that of exhausted T cells in chronic viral infections and cancers, suggesting a link between T cell exhaustion and autoimmunity (Tilstra et al., 2018).

For this reason, the current data of differentially expressed genes between IFN-γ SP and DP cells, sorted from memory CD4 T cells of diseased NZBxW lupus-prone mice were compared with differentially expressed genes between memory, effector and exhausted CD4 T cells, isolated from mice with chronic infection with lymphocytic choriomeningitis virus (LCMV) from the work of Crawford et al. (Crawford et al., 2014). This proceeding revealed that the gene profile of IFN-γ SP cells of diseased NZBxW lupus-prone mice possessed a significant overlap with that of exhausted T cells and to a lesser extent memory T cells of the model of chronic viral infection. Hence, these findings point to a status of the IFN-γ SP cell subset that shares features with exhausted T cells.

T cell exhaustion during chronic viral infection is associated with suppression of immune responses through increased expression of co-inhibitory receptors as well as a progressive loss of effector functions, which results in viral persistence (Wherry, 2011). However, the “exhaustion signature” was shown to be associated with a better clinical outcome in hepatitis C virus (McKinney et al., 2015). In contrast, it exhibited a poor outcome in several autoimmune diseases (among others SLE) (McKinney et al., 2015). The authors speculated that the clinical outcome might be dependent on the balance of co-inhibitory and co-stimulatory receptors expressed on T cells.

Since IFN-γ SP cells of diseased NZBxW lupus-prone mice were characterized by increased expression of co-inhibitory receptors (CTLA-4, LAG-3) and increased apoptosis potential along with decreased proliferation and decreased expression of effector cytokines (IL-2, TNF-α), they might have reduced functionality and resemble “exhausted” T cells. On the other side, IFN-γ SP

cells displayed increased expression of inflammatory chemokine receptors (CCR2, CCR5, CXCR4, CXCR6), increased expression of co-stimulatory receptors (CD27, CD40L) and increased expression of pro-inflammatory cytokines (IFN-γ and IL-10), which might evoke pro-inflammatory activity and consequently, persistence of inflammation, establishing them as "hyperactivated" T cells. Taken together, IFN-γ SP cells of diseased NZBxW mice share features of "exhausted" and "hyperactivated" cells and assignment of these cells to only one or another state is not conclusive on the basis of current data, yet.

4.5. Differentiation of IFN-γ SP cells upon IFN-γ administration to the *in vitro* cultures

The next aim of this work was to analyze stability of cytokine production within distinct subsets of memory CD4 T cells (DN, IL-2 SP, DP and IFN-γ SP cells) using *in vitro* cultivation of cells. For interpretation of the results, the conditions of cell cultivation are to be considered. Prior to cultivation, non-physiological stimulation of cells with PMA and Ionomycin was necessary for induction of cytokine production and subsequent separation of cells using cytokine secretion assay. In the culture, recombinant cytokines IL-2, IL-7 and IFN-γ were added. Administration of IL-2 and IL-7 to the cultures was necessary to provide proliferation and survival signals, respectively (Boyman and Sprent, 2012; Bradley et al., 2005). Administration of IFN-γ was necessary to ensure a balanced and foremost comparable cytokine milieu between the isolated subsets of memory CD4 T cells.

Under these conditions, DN cells gained the ability to produce IFN-γ with a fraction becoming IFN-γ SP cells. A fraction of DP cells in their turn lost the ability to produce IL-2, but continued to produce IFN-γ, thus becoming IFN-γ SP cells, as well. IFN-γ SP cells themselves were stable in IFN-γ production over time. In contrast, a fraction of IL-2 SP lost the ability to produce IL-2, however, these cells did not gain the ability to produce IFN-γ even in the presence of IFN-γ supplementation. Remarkably, these observations indicate that under certain conditions of repetitive stimulation DN and DP cells, but not IL-2 SP cells might have the ability to differentiate into IFN-γ SP cells.

IFN-γ provides signals that foster the T_H1 phenotype and therefore its own expression (Bradley et al., 1996). Becattini et al. showed that 45-50% CD4 naive IFN-γ SP ($IL\text{-}17^{-}IL\text{-}4^{-}$) cells produced

IFN-γ on day seven after stimulation with a pathogen (Becattini et al., 2015). In addition, Helmstetter et al. showed that the pool of T_H1 cells, generated during LCMV infection *in vivo*, exhibited a quantitative memory for IFN-γ production and both the amount of IFN-γ per cell and the probability for a cell to produce IFN-γ are stable over time (Helmstetter et al., 2015).

Thus, one could hypothesize that the three subsets of memory CD4 T cells (DN, DP and IFN-γ SP cells), that showed the ability to become IFN-γ SP cells *in vitro* might be programmed to produce IFN-γ in diseased NZBxW lupus-prone mice. It needs to be considered that administration of IFN-γ during *in vitro* culture may have influenced the direction of cell differentiation towards IFN-γ production (Smeltz et al., 2002). However, IFN-γ is expected to be available *in vivo*, especially in cases of ongoing autoimmunity, like in diseased NZBxW lupus-prone mice. Therefore, it needs to be determined, how IFN-γ SP cells develop *in vivo* and whether they arise from DN and/or DP memory CD4 T cells.

4.6. Impact of stimulation of cells with PMA and Ionomycin

Production of cytokines by T cells is largely dependent on activation. Under physiological conditions, T cells are activated through the recognition of specific antigen by the T cell receptor (TCR) and only in combination of co-stimulatory signals, both provided by APCs (Smith-Garvin et al., 2009). To stimulate the whole population of T cells in an antigen-independent way, chemical treatment with phorbol 12-myristate 13-acetate (PMA) and Ionomycin is widely used in the laboratory. PMA activates Protein kinase C (PKC) and Ionomycin induces calcium influx into the cell, thereby initiating the main signal transduction axes of TCR stimulation. In this way, stimulation-associated transcription factors get activated and induce the gene expression program of T cell activation (e. g. cytokines) (Hashimoto et al., 1991).

However, stimulation of T cells with PMA and Ionomycin leads to maximal cytokine expression, since it is several-fold stronger than the physiological stimulation. On the one hand, full stimulation was well suitable in this work for maximal cytokine expression; on the other hand, a number of observed alterations in gene expression might not reflect the status under physiological conditions, even under continuous antigen exposure. Therefore, the expression pattern of putative disease-associated gene candidates was verified on RNA and protein levels in *ex vivo* isolated memory CD4 T cells of NZBxW lupus-prone mice with high disease activity or

no disease activity as defined by clinical parameters, such as age, weight, proteinuria, lymphadenopathy and splenomegaly.

For instance, it was found that IFN-γ SP cells of disease mice produced higher frequencies of IL-10, when compared to the young mice before onset of the disease, suggesting that the ability to express IL-10 was acquired with disease manifestation rather than caused by artificial stimulation. In addition to IL-10, alterations in the expression of several other markers, such as TNF-α, GITR, CD27, LAG-3 and CTLA-4 before and after onset of lupus nephritis were also verified (not all data are shown). As all cells were equally stimulated with PMA and Ionomycin, the observed changes revealed the impact of disease activity on the gene expression profile.

Conclusions

Multi-step sorting of memory CD4 T cells using flow cytometry allowed for isolation of four cell subsets: DN, IL-2 SP, DP and IFN-γ SP cells. This kind of cell purification enabled informative gene expression analyses of defined cell subsets. In contrast to DP cells, IFN-γ SP cells of diseased NZBxW lupus-prone mice were characterized by increased expression of inflammatory chemokine receptors (*Cxcr6, Cxcr4, Ccr8, Ccr5, Ccr2, Ccr9*), increased expression of co-inhibitory receptors (*Lag3, Ctla2a, Cd80, Ctla4*), increased expression of co-stimulatory receptors (*Cd27, Cd40lg*), increased expression of granzymes (*Gzma, Gzmb*), increased expression of IFN-regulated transcription factors (*Stat1, Irf1,Irf9, Smad2*), and decreased cytokine expression (*Il9, Tnf, Ifng, Il18, Il17a, Il17f, Il2, Il5, Il3)*, except increased expression of *Il10.* In addition, functional assays showed that in contrast to DP cells, IFN-γ SP cells in diseased NZBxW lupus-prone mice tended to increased apoptosis and decreased proliferation. Comparison of gene expression data of IFN-γ SP memory T cells of diseased NZBxW lupus-prone mice with literature data showed a significant overlap with genes, which were expressed in memory and exhausted CD4 T cells during chronic viral infections.

Specific gene expression pattern of IFN-γ SP cells of diseased NZBxW lupus-prone mice might have potential pathogenic features upon aberrant expression of many pro-inflammatory molecules. However, current experiments do not discover the precise role of individual genes and their network in SLE, therefore the interpretation of the entire data is limited at this time point. Nevertheless, enhanced expression of chemokine receptors and co-stimulatory molecules on IFN-γ SP cells might be responsible for their enhanced activation, survival and maintenance, pinpointing them as “hyperactivated” cells, which might be responsible for the persistence of inflammation. From the other sight, increased expression of co-inhibitory receptors, apoptosis markers as well as decreased proliferative capacity and decreased production of effector cytokines (especially the loss of the ability to produce IL-2 and TNF-α) might point to their state of short-lived “terminally differentiated” or “exhausted” cells.

Outlook

This work opens up further issues for research in the field of autoimmunity and particularly in SLE. First, the gene expression data of the isolated subpopulations of memory CD4 T cells of diseased NZBxW lupus-prone mice can provide information for more target-oriented research with regard to differentially expressed molecules. Moreover, the data of this work point to the hypothesis that the IFN-γ SP cell subset of memory CD4 T cells might be the source of aberrant IFN-γ production and therefore, drivers of the pathogenesis of SLE and the permanent persistence of inflammation in NZBxW lupus-prone mice. To prove this hypothesis, adoptive-transfer experiments of isolated subsets of memory CD4 T cells (DN, IL-2 SP, DP and IFN-γ SP cells) are needed. They will shed light not only on the role of distinct memory CD4 T cell subsets in SLE pathogenesis, but they will also give information about the direction of memory CD4 T cell differentiation and development in SLE.

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Declaration of Originality

I declare herewith that I am the sole author of this work and composed it without any assistance. This work has not been published. All sources and methods used in this work are clearly referenced.

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